Investigating the Role of Deoxyhypusine Synthase in the Invasiveness of PC3 Cells Using siRNA

by

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A thesis
presented to the University of Waterloo
in fulfillment of the
thesis requirement for the degree of
Master of Science
in
Biology

Waterloo, Ontario, Canada, 2008

AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Abstract

Deoxyhypusine synthase (DHS) catalyzes the first step in the hypusination of eukaryotic translation initiation factor 5A (eIF5A). In human cells, two eIF5A isoforms are present, eIF5A-1 and eIF5A-2, and DHS catalyzes the hypusination of both. Since both eIF5As are substrates for DHS, the biological functions of DHS are likely to be exerted through the various post-translational forms of these two eIF5As. The lysine form of eIF5A-1 has been associated with apoptosis, while the hypusinated form of eIF5A-1 has been associated with cell viability and proliferation. eIF5A-2 has been found to be over-expressed in certain cancers and has been proposed to function as an oncogene. *Dhs* is also over-expressed in certain human cancers and is a metastatic signature gene.

The purpose of the present study was to investigate the role of DHS in cancer cell invasiveness, cell proliferation, and apoptosis using RNA interference. The main finding of the study is that DHS siRNA treatment decreases invasiveness of PC3 cells *in vitro*. Both DHS 0 siRNA treatment and DHS 1/b siRNA treatment significantly reduced cell invasiveness of PC3 cells as measured by the Matrigel invasion assay. Potential confounding variables, such as differences in cell proliferation or differences in apoptosis in response to DHS siRNA treatment, were assessed using the XTT cell proliferation assay and the Annexin V/Pi apoptosis assay, and they were found not to have an effect. In the absence of serum, DHS siRNA treatment did not result in significant decrease in cell proliferation compared to the control siRNA treatment. Furthermore, DHS siRNA treatment did not induce apoptosis in PC3 cells under the present experimental conditions. In conclusion, depletion of DHS with RNAi reduces invasiveness, but does not induce apoptosis in PC3 cells. The significance of

the research is that the anti-invasiveness effect of DHS depletion in metastatic cancer cells is shown for the first time in the present study. Thus, DHS depletion may be useful to combat cancer in conjunction with L-eIF5A-1 over-expression.

Acknowledgements

I am grateful to my supervisor, Dr. Thompson, and to everyone in the Thompson Lab who helped me with useful suggestions. I am also indebted to Richard Dondero from Senesco Technologies, Inc. for his helpful comments. I thank my committee members for their patience and for reading my thesis. I also wish to express my gratitude for the financial assistance I received from NSERC.

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Chapter 1
Introduction

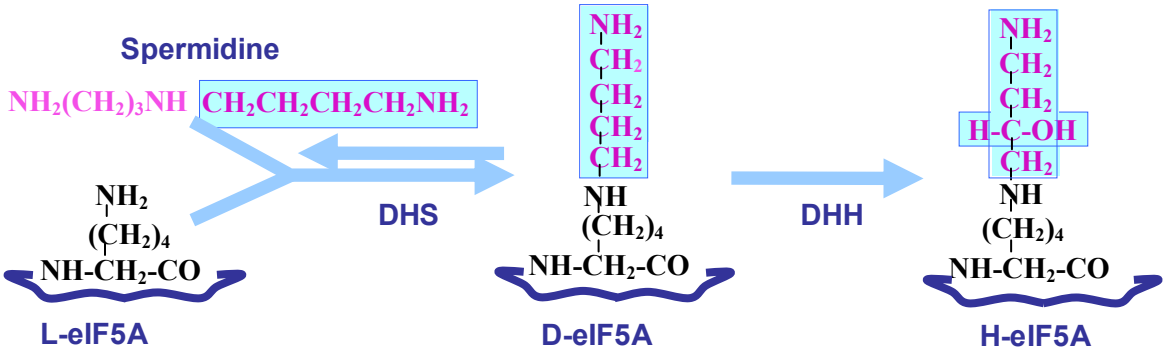
Deoxyhypusine synthase (DHS, a.k.a. DHPS) is an important enzyme in the hypusination pathway of eukaryotic translation initiation factor 5A (eIF5A) (Figure 1) (Park, 2006; Klier et al., 1995). Hypusine (N^{ε}-(4-amino-2-hydroxybutyl) lysine), a rare post-translational modification, has only been found in a single protein, eIF5A (Park, 2006). Hypusine, eIF5A, and DHS are necessary for cell viability and also play a role in cell proliferation in eukaryotic cells (Park, 2006; Wolff et al., 2007). *Dhs* has also been found to be a metastatic signature gene (Ramaswamy et al., 2003). The specificity of the hypusination reaction, the role of the hypusinated eIF5A and DHS in cell proliferation, and DHS up-regulation in metastatic cells suggest that the hypusination pathway presents several opportunities for intervention in aberrant cell proliferation and cancer progression (Wolff et al., 2007). The present research focuses on human DHS as a potential target for intervening in cell proliferation and metastasis using RNA interference.

1.1 Deoxyhypusine synthase

DHS was first discovered in 1987 when Murphey and Gerner were able to show that the hypusination of eIF5A was catalyzed by two enzymes that had different optimal pH values. This finding suggested that another enzyme, distinct from the already known deoxyhypusine hydroxylase, catalyzes the first step of eIF5A hypusination (Murphey and

Figure 1. The hypusination pathway of eIF5A.

L-eIF5A is converted to the D-eIF5A intermediate by DHS, and subsequently, D-eIF5A is converted to H-eIF5A by DHH. In the first step, the 4-aminobutyl moiety of spermidine is transferred to $Lysine_{50}$ of L-eIF5A. Abbreviations: L-eIF5A, eukaryotic translation initiation factor 5A precursor with $Lysine_{50}$(in humans); D-eIF5A, eIF5A intermediate containing deoxyhypusine at $Lysine_{50}$; H-eIF5A, hypusinated eIF5A containing hypusine at $Lysine_{50}$; DHS, deoxyhypusine synthase; DHH, deoxyhypusine hydroxylase (Modified from Park, 2006).


Spermidine
NH2(CH2)3NH
CH2CH2CH2CH2NH2
DHS
DHH
L-eIF5A
D-eIF5A
H-eIF5A

Gerner, 1987). Park et al. (2003) demonstrated that the first step of the deoxyhypusine synthesis reaction, catalyzed by DHS, is reversible (Figure 1).

DHS, like eIF5A, is well conserved through evolution (Wolff et al., 2007). DHS is present in all eukaryotes, in certain archaea, and even several cyanobacteria contain a DHS cognate (Wolff et al., 2007). In most eukaryotes, DHS is encoded by a single gene, although a homolog gene that encodes homospermidine synthase—presumably created from the DHS gene by gene duplication—has also been found in certain plants (Wolff et al., 2007).

As most known DHS-containing eukaryotic organisms, humans also have only one DHS gene (Wolff et al., 2007). The human DHS (DHPS) gene (Gene ID: 1725; GenBank accession number U79262) (a.k.a. migration-inducing gene 13) is located on the short arm of chromosome 19 (19p13.11-p13.12) (Jones et al., 1996). The gene consists of nine exons extending across 6.6 kb. Three transcript variants have been isolated so far: (1) splice variant 1 (GenBank accession number NM_001930.2), which includes exon 7 and alternative exon 8a, encodes *isoform a* of the enzyme (GenPept accession number NP_001921.1); (2) splice variant 2 (GenBank accession number NM_013406.1), which lacks exon 7 but includes alternative exon 8b, encodes *isoform b* (GenPept accession number NP_037538.1); and (3) splice variant 3 (GenBank accession number NM_013407.1), which lacks exon 7 and includes alternative exon 8a, encodes *isoform c* (GenPept accession number NP_ 037539.1). Of the three transcript variants, only splice variant 1, *isoform a*, encodes a protein that has deoxyhypusine synthase activity; whereas, the other two transcript variants may function as modulating factors of DHS activity (Joe and Park, 1995).

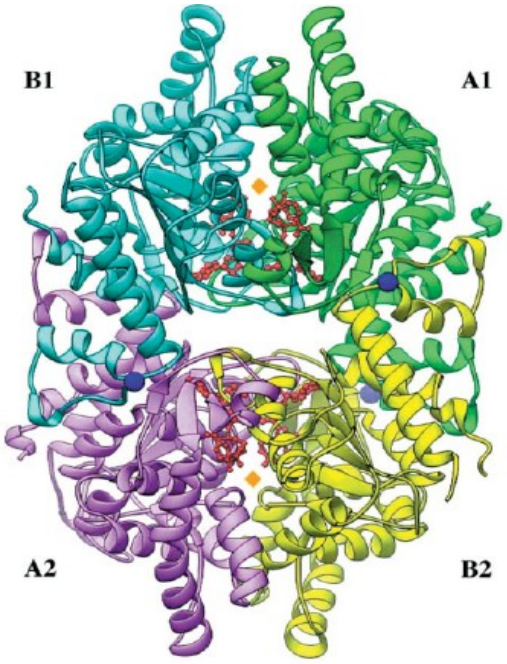
Human DHS is a homotetramer with an apparent molecular weight of 150 kDa and with each subunit weighing approximately 40kDa (Klier et al., 1995). The four subunits are organized into two tightly associated dimers, each of which contains two spermidine-biding sites, amounting to four spermidine-binding sites in total (Figure 2) (Park, 2006). The interconversion between the active and inactive form of the enzyme is mediated by a ball-and-chain motif, an N-terminal two-turn α helix structure (Park, 2006). The catalytically active site, K_{329}, and four of the five key amino acid residues (Asp_{243}, His_{288}, Asp_{316}, Glu_{323}, and Trp_{327}) are essential for spermidine binding and DHS activity, and they are strictly conserved among eukaryotes (Figure 2) (Wolff et al., 2007). DHS has also been found to have hidden stereospecificity, as Hyvonen et al. (2007) has shown that between (*S*) and (*R*) stereoisomers only (*S*)-methyl-spermidine was converted to hypusine.

The most potent chemical inhibitors of DHS activity discovered so far are N^1-guanyl-1,7-diamino-heptane (GC7) and its methyl derivative (Park et al., 1994; Park, 2006; Lee et al., 2002). GC7 competitively binds to the DHS active site, and it has been reported to have high affinity and high selectivity for DHS (Lee et al., 2002). However, Chen et al. (1996) pointed out that "the possibility that GC7 may compete with spermidine in other physiological events" cannot be excluded, and thus, DHS may be a better target for interfering with cancer (Chen et al., 1996). Furthermore, several diamine and triamine derivatives were found to inhibit DHS to a lesser degree than GC7, and their effects on growth arrest cannot be attributed to their inhibitory effect on DHS and hypusine formation (Lee at al., 1995). Similarly, Taylor et al. (2007) also suggested that

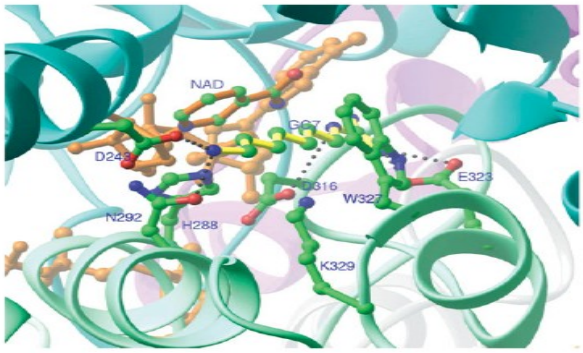
Figure 2. DHS crystal structure and its active site.

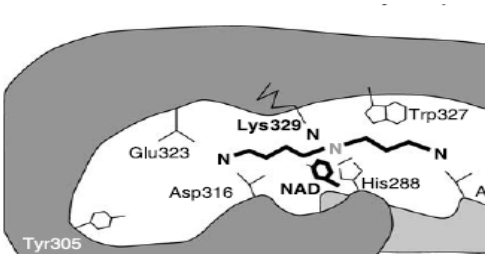
A. DHS·NAD tetramer, showing Form II, with each diamond shape indicating an active site. The total number of active sites for the enzyme is four (Umland et al., 2004). B. Proposed mode of spermidine binding in the active site of the DHS·NAD complex (Wolff et al., 2007; Umland et al., 2004). The catalytically active site is Lys_{329}, and four of the five key amino acid residues, Asp_{243}, His_{288}, Asp_{316}, Glu_{323}, and Trp_{327}, are essential for spermidine binding and DHS activity. Top Panel: Active site showing spermidine binding; Bottom Panel: schematic diagram of the enzyme complex emphasizing the key amino acids in the active site.

A


B1
A1
A2
B2

B


NAD
D243
N292
H288
K329
E323


Lys329
Trp327
Glu323
Asp316
NAD
His288
Tyr305

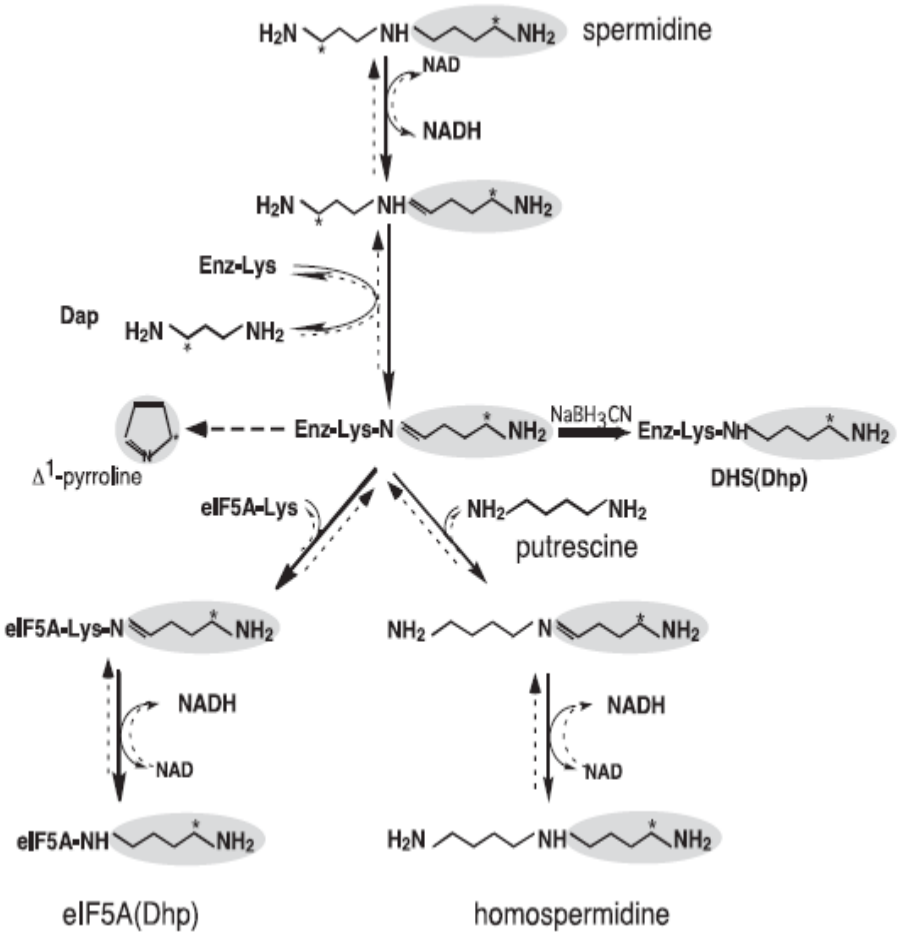
GC7 may not be a specific inhibitor since when they depleted all forms of eIF5A-1 in HT-29 cells (more than 90% depletion levels were achieved) no effect on cell proliferation was observed, while when they treated cells with 50 μM GC7 for 72 hours, cell proliferation decreased significantly compared to the control siRNA-treated cells. In addition, Park (2006) also pointed out that although DHS seems to be the only target of GC7, and GC7 does not seem to interfere with other enzymes of polyamine biosynthesis and metabolism, it remains possible that GC7 has some negative side effects. Consequently, targeting DHS with RNAi could provide an equally potent, but more specific inhibitory effect on enzyme function with fewer side effects.

The DHS enzyme (EC 2.5.1.46) plays a crucial role in the hypusination of eIF5A, since DHS catalyzes the first step in the hypusination pathway of eIF5A (old nomenclature eIF-4D) (Figure 1) (Wolff et al., 2007). Hypusine is a modified lysine, (N^{ϵ}-(4-amino-2-hydroxybutyl) lysine), which was named after its two structural components: hydroxyputrescine and lysine (Shiba et al., 1971). Hypusination of eIF5A takes place in two enzymatic steps: in the first, reversible step, DHS catalyzes the transfer of the 4-aminobutyl moiety of the polyamine spermidine to the ε-amino group of $Lysine_{50}$ (in humans) in the eIF5A precursor, L-eIF5A, to form an intermediate, deoxyhypusine (D-eIF5A) (Figure 1, Figure 3). Subsequently, deoxyhypusine is hydroxylated to hypusine by another enzyme, deoxyhypusine hydroxylase (DHH, a.k.a. DOHH), resulting in the mature hypusinated form of eIF5A (H-eIF5A) (Figure 1) (Park et al., 1994).

Deoxyhypusine synthesis itself takes place in four separate steps: first, spermidine undergoes dehydrogenation to dehydrospermidine in an NAD-dependent manner; second, the 4-aminobutyl moiety from dehydrospermidine is cleaved

Figure 3. Mechanism of the DHS catalyzed reaction.

Deoxyhypusine synthesis takes place in four steps: NAD-dependent dehydrogenation of spermidine, transfer of the 4-aminobutyl moiety to the ε-amino group of $Lysine_{329}$ (in humans) of DHS, transfer of the 4-aminobutyl moiety to the ε-amino group of $Lysine_{50}$ (in humans) of eIF5A, and the reduction of the eIF5A-imine intermediate to D-eIF5A (Park, 2006). Depending on substrate availability, DHS can catalyze the transfer of the 4-aminobutyl moiety to either L-eIF5A, putrescine, or 1,3-diaminopropane through an enzyme-imine intermediate. In the absence of an acceptor, the 4-aminobutyl moiety of the enzyme-imine intermediate is cyclized to Δ^1-pyrroline (Park, 2006).


spermidine
NAD
NADH
Enz-Lys
Dap
Enz-Lys-N
NaBH3CN
Enz-Lys-NH
DHS(Dhp)
Δ1-pyrroline
eIF5A-Lys
putrescine
eIF5A-Lys-N
NADH
NAD
eIF5A-NH
eIF5A(Dhp)
NADH
NAD
homospermidine

and transferred to the ε-amino group of an active site lysine residue ($Lysine_{329}$ in humans) and forms an enzyme-imine intermediate; third, the 4-aminobutyl moiety from the enzyme intermediate is transferred to the ε-amino group of a $Lysine_{50}$ (in humans) of L-eIF5A, and forms an enzyme-eIF5A intermediate; and finally, enzyme-bound NADH reduces the eIF5A-imine intermediate to form a deoxyhypusine residue (Figure 3) (Park, 2006).

In addition to using L-eIF5A as a substrate, human DHS can also use putrescine as a butylamine acceptor in case L-eIF5A is not available; nevertheless, DHS has a higher affinity to L-eIF5A than for putrescine, clearly preferring the hypusination reaction (Figure 3) (Park, 2006). Furthermore, the DHS reaction in the hypusination pathway of eIF5A is reversible (Figure 1, Figure 3) (Park et al., 2003). Thus, DHS can transfer the aminobutyl moiety of the enzyme-imine intermediate to any of three acceptors: L-eIF5A producing D-eIF5A, putrescine producing homospermidine, or 1,3-diaminopropane producing spermidine (Figure 3) (Park et al., 2003). When none of the potential acceptors are present, the 4-aminobutyl moiety of the enzyme-imine intermediate is cyclized to form Δ^1-pyrroline (Park, 2006). In sum, DHS can catalyze the interconversion of spermidine, D-eIF5A, and homospermidine (Park, 2006). However, the strong preference of DHS toward L-eIF5A creates a specificity that can be used to intervene in cellular processes associated with the hypusination of eIF5A (Wolff et al., 2007).

Since the primary function of DHS is the catalysis of the first step of the hypusination pathway of eIF5A, it is indirectly involved in the functions attributed to eIF5A. Despite more than twenty years of intensive study, however, the precise function of eIF5A has not been elucidated (Wolff et al., 2007). eIF5A has two isoforms in the human genome, eIF5A1 and eIF5A2, which are 84% identical and 94% similar at the amino acid level (Jenkins et al., 2001).

DHS has been found to interact with both isoforms, and thus, eIF5A2 also contains the hypusine modification (Clement et al., 2006; Jenkins et al., 2001; Rual et al., 2005). Whereas eIF5A1 is constitutively expressed in most mammalian cells, eIF5A-2 has been found to be expressed at a high level only in brain and testis and in certain ovarian and colorectal cancers, although eIF5A-2 mRNA has been found in all human cells (Clement et al., 2006; Guan et al., 2001; Jenkins et al., 2001). *eIF5A-2* is located at 3q25-27, a region known for chromosomal instability in cancers, and in accordance with its signature over-expression in certain cancers, *eIF5A-2* was suggested to function as an oncogene (Clement et al., 2006; Guan et al., 2001).

1.2 Functions of eIF5A

eIF5A-1 has been proposed to have various functions. In yeast, eIF5A and its hypusine modification have been found to be essential for cell viability (Kang and Hershey, 1994). eIF5A-1 has been found to be important in cell proliferation in human and mammalian cells (Jasiulionis et al., 2007; Park et al., 1994; Kang and Hershey, 1994). H-eIF5A has been found to stimulate the first step in protein synthesis, the reaction of methionyl-$tRNA_f$ in 80S initiation complexes with puromycin *in vitro*, and thus, it was concluded that H-eIF5A was essential for protein synthesis (Benne and Hershey, 1978). However, Kang and Hershey (1994) found that eIF5A-depleted cells still synthesized protein at about 70% of the wild type rate, and thus concluded that eIF5A may be necessary only for the translation of a selected subset of mRNAs or may be involved in some other aspects of cell metabolism. Using affinity co-purification, Xu et al. (2004) identified 20 RNA sequences that co-purified with eIF5A, including RNAs encoding ribosomal L35a, plasminogen activation inhibitor mRNA-binding protein, NADH dehydrogenase subunit and ADP-ribose pyrophosphatase. In addition, eIF5A has been found to

functionally mediate *Rev* expression, the viral trans-activator protein necessary for human immunodeficiency virus type 1 by mediating its export in conjunction with a nuclear export protein (Ruhl et al., 1993). Lipowsky et al. (2000) found that H-eIF5A was transported from the nucleus by Exportin 4. Zuk and Jacobson (1998) have shown that eIF5A plays a role in mRNA turnover, and Valentini et al. (2002) conducted experiments with yeast mutants that confirmed eIF5A's involvement in mRNA turnover. Moreover, they further suggested that eIF5A may also be involved in ribosomal synthesis and cell wall integrity (Valentini et al., 2002). Additionally, Zanelli and Valentini (2005), working with yeast, showed that eIF5A is important for establishing actin polarity, which is essential for G1/S transition in yeast. Further, eIF5A has been suggested to function as a sensor of intracellular polyamine levels to regulate the growth status of mammalian cells in culture by modifying the translation of a subset of growth-related mRNAs (Jenkins et al., 2001). Finally, Taylor et al. (2004) showed that TNF-α-induced apoptosis resulted in eIF5A up-regulation, and siRNA-mediated downregulation of eIF5A protected human lamina cribrosa cells (involved in glaucoma) from apoptosis. Taylor et al. (2007) also showed that L-eIF5A induces apoptosis in colon cancer cells, and thus they suggested that L-eIF5A may have a pro-apoptotic function. In sum, many different, seemingly disjointed, functions have been proposed for eIF5A, but a conclusive, overarching theory that explains the diverse experimental results has not been put forward yet.

In addition to its role as a catalyst of the eIF5A hypusination pathway, DHS has also been proposed to have a regulatory role in eIF5A hypusination, or to have another, so far unknown, independent cellular function (Kang and Chung, 2003; Kang et al., 2002). The evidence for these roles, however, has been inconsistent so far. First, deoxyhypusine synthase has been shown to be

phosphorylated by protein kinase C (PCK) in yeast (Kang and Chung, 1999). Similarly, Kang et al. (2002) showed that DHS is phosphorylated by PCK in a phorbol 12-myristate 13 acetate (PMA) negative, and Ca^{2+}/phospholipid independent manner in various cell lines such as Chinese Hamster Ovary (CHO), NIH3T3 (mouse fibroblast cell line), and chicken embryonic cells. Second, DHS has also been found to be phosphorylated by protein kinase CK2 on threonine and serine residues in human HeLa cells (Kang and Chung, 2003). Based on these results and its strategic position as the enzyme that catalyzes the first, reversible step in the hypusination pathway of eIF5A, DHS was proposed to play a regulatory role in eIF5A hypusination. However, when measuring DHS enzyme activity of the phosphorylated and the unphosphorylated forms of DHS, Kang and Chung (2003) did not find a difference, and thus, they concluded that DHS may play a role in another, independent cellular function as well. Third, using deletion studies in yeast, Thompson et al. (2003) have shown that the N-terminal α-helix of DHS can regulate DHS's interaction with its substrate, L-eIF5A. These results suggest that the various domains of eIF5A have independent binding sites in DHS, and that the N-terminal α-helix modulates eIF5A-$Lysine_{51}$ (in yeast) access to the active site. Consequently, Thompson et al. (2003) proposed a regulatory mechanism where inactive DHS under negative control of the N-terminal α-helix opens the catalytic site to L-eIF5A only when H-eIF5A is needed, in case of cell cycle transition, for instance. Thus, although the evidence so far is contradictory, DHS may play a key regulatory role in the hypusination of eIF5A perhaps under the control of one of the various cell proliferation signal transduction pathways (Thompson et al., 2003).

1.3 Cell proliferation and deoxyhypusine synthase

Cell proliferation, or an increase in cell number, is the result of the process of cell content duplication and cell division known as the cell cycle (Alberts *et al*., 2002). Progression through the cell cycle is regulated by a complex network of regulatory proteins called the cell-cycle control system, which controls cell numbers in the tissues of the body of multicellular animals, including humans (Alberts *et al*., 2002). The fundamentals of the cell cycle and its control system are the same in all eukaryotic cells. When the cell-cycle control system breaks down, excessive cell proliferation can result in cancer (Alberts *et al*., 2002).

DHS has been found to be involved in cell proliferation via its catalytic role in the hypusination pathway of eIF5A (Park, 2006). The hypusinated form of eIF5A has been found to be important in cell proliferation and cell viability in numerous studies (Park et al., 1994; Park, 2006). For example, Park et al. (1994) have tried various guanyl diamines as DHS inhibitors, and they found that the most potent DHS inhibitor was GC7, showing the highest antiproliferative effect *in vitro* on Chinese Hamster Ovary (CHO) cells. They also found that the reversible inhibitory effect of GC7 was not due to a change in polyamine metabolism, but to the inhibitory effect on eIF5A hypusination (Park et al., 1994). Subsequently, treatment with GC7 has been found to produce proliferation arrest in several different mammalian and human cancer cells (Park et al., 1994; Lee and Folk, 1998). For example, Lee et al. (2002) found that GC7 caused dose-dependent inhibition of hypusine formation and cell proliferation in human umbilical vein endothelial cells (HUVEC), with a lower dose (10μM) causing complete hypusine synthesis inhibition and cytostasis, whereas pre-treatment (5-50μM for 96 hours) with GC7 protected cells from serum starvation-induced apoptosis. In both cases, DHS-inhibition resulted in the

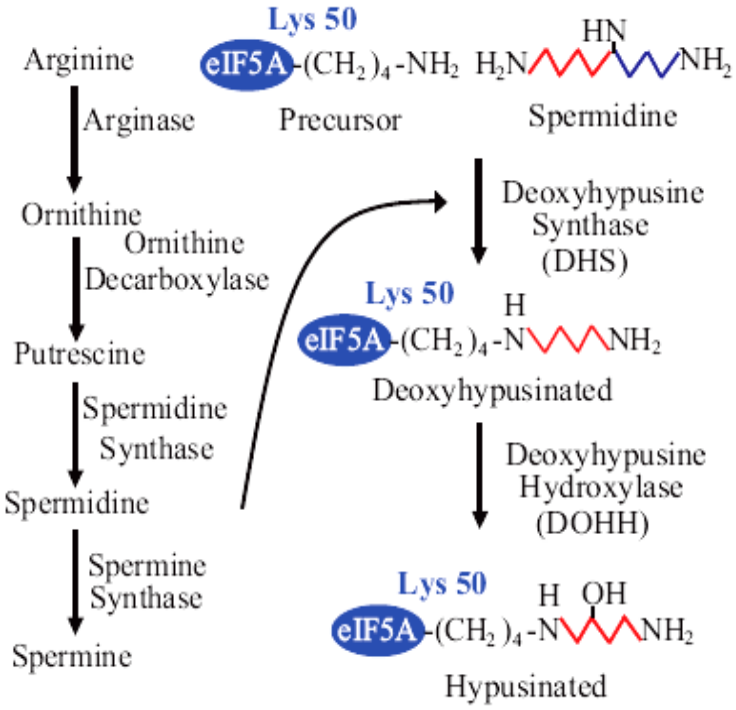
accumulation of the eIF5A precursor protein (Lee *et al*., 2002). Thus, Lee et al. (2002) concluded that eIF5A may be involved in the expression of proteins needed for both cell proliferation and apoptosis.

Since in the first step of eIF5A hypusination DHS uses spermidine as a 4-butylamine donor, the eIF5A hypusination pathway is also connected to polyamine biosynthesis (Figure 4). Polyamines, such as putrescine, spermidine and spermine, are necessary for cell proliferation as well as cell growth, and spermidine is also used for the hypusination of eIF5A (Nishimura et al*.,* 2005). Polyamines have been found to exert their proliferative effect through stimulation of protein synthesis (Nishimura et al*.,* 2005). Initially polyamine depletion was believed to result in arrest in cell proliferation through its effect on H-eIF5A (Nishimura et al*.,* 2005). However, when Nishimura et al*.,* (2005) selectively inhibited DHS with GC7 without affecting polyamine levels, H-eIF5A level decreased on day 1, but cell proliferation inhibition was observed only from day 2, suggesting that the two effects are likely to be independent.

By contrast, when ornithine decarboxylase was inhibited with DFMO (α-difluoromethylornithine) or with DFMO and spermine synthase with APCHA (N^1-(3-aminopropyl)-cyclohexylamine) together, cell growth was inhibited on day 1, but the level of H-eIF5A started to decrease only after 12 hours, albeit DFMO effectively decreased the levels of putrescine and spermidine (Nishimura et al*.,* 2005). Thus, Nishimura et al*.,* (2005) showed that

Figure 4. Polyamine biosynthesis and the eIF5A hypusination pathway.

In the first step of the hypusination pathway of eIF5A, spermidine is the source of the 4-aminobutyl moiety that is transferred to L-eIF5A. Thus the hypusination pathway is connected to the polyamine biosynthesis pathway. Tracing the polyamine pathway backwards, spermidine is synthesized from putrescine by spermidine synthase; putrescine is synthesized from ornithine by ornithine decarboxylase; and putrescine is synthesized from arginine by arginase. Spermidine is also the substrate for spermine synthase which catalyzes the production of spermine (Huang et al., 2007).


Lys 50
eIF5A-(CH2)4-NH2
Precursor
HN
H2N
NH2
Spermidine
Arginine
Arginase
Ornithine
Ornithine Decarboxylase
Putrescine
Spermidine Synthase
Spermidine
Spermine Synthase
Spermine
Deoxyhypusine Synthase (DHS)
Lys 50
eIF5A-(CH2)4-N
H
NH2
Deoxyhypusinated
Deoxyhypusine Hydroxylase (DOHH)
Lys 50
eIF5A-(CH2)4-N
H
OH
NH2
Hypusinated

eIF5A and polyamines are independently involved in cell proliferation; nevertheless, DHS acts at the connection between these two pathways.

1.4 Apoptosis and deoxyhypusine synthase

Apoptosis, or programmed cell death, is an orderly way of cell suicide in multicellular organisms (Alberts *et al*., 2002). Besides being very important during development, apoptosis is also crucial in adult organisms, where it is necessary to counterbalance cell proliferation and ensure the constant size of the organism (Alberts *et al*., 2002). In adults, apoptosis is also necessary to dispose of cells that are no longer required, immune cells after clearing out an infection, for instance, and to defend against cancer since cells with irreparable genetic damage that have the potential to develop into cancerous cells are also killed by apoptosis (Karp, 2002).

DHS plays an indirect role in apoptosis via its catalytic role in the hypusination pathway of eIF5A. The most potent competitive inhibitor of DHS, GC7, has been found to induce five to six times as much apoptosis as the controls in PC-12 cells, a cell line derived from pheochromocytoma, a neuroendocrine tumor of the adrenal gland, from *Rattus norvegicus* (Huang et al., 2007). Furthermore, to test for possible non-specific toxicity of GC7, Huang et al. (2007) also used small interfering RNAs (siRNAs) against DHS, and they found that DHS siRNA-treated cells showed a similar level of apoptosis as GC7-treated cells. Consequently, they concluded that apoptosis could not have been caused by nonspecific cytotoxicity from either GC7 or DHS siRNAs because the control, scrambled siRNA did not cause comparable apoptosis (Huang et al., 2007). They confirmed that hypusination of eIF5A was necessary for cell survival, and since both GC7 and DHS siRNA blocked eIF5A hypusination without affecting polyamine

synthesis, their affect on apoptosis was attributed to blocking H-eIF5A formation (Huang et al., 2007).

Similarly, Taylor et al. (2007) found supporting evidence for the pro-apoptotic role of the unhypusinated L-eIF5A, suggesting that inhibition of DHS activity might result in the induction of apoptosis via the accumulation of the L-eIF5A. They found that over-expression of eIF5A led to apoptosis in HT-29 colon adenocarcinoma cells by possibly overwhelming the capacity of DHS to hypusinate L-eIF5A, thus resulting in the accumulation of L-eIF5A (Taylor et al., 2007). Likewise, Taylor et al. (2007) also found that over-expression of a mutant form of eIF5A, which could not be hypusinated by DHS, resulted in the accumulation of L-eIF5A and apoptosis. Thus, Taylor et al. (2007) concluded that L-eIF5A has a pro-apoptotic function. Consequently, inhibition of DHS activity with inhibitors, such as GC7, which have been found to result in apoptosis (Chen et al., 1996; Huang et al., 2007; Park et al., 1998), would likely to be the result of the accumulation of L-eIF5A, which has a pro-apoptotic function (Taylor et al., 2007).

Conversely, other researchers found evidence for DHS and hypusination of eIF5A being necessary for apoptosis. Li et al. (2004) found that DHS is involved in H-eIF5A-mediated apoptosis through eIF5A's function as a regulator of p53 independently from eIF5A's role in protein translation. Using yeast two-hybrid screens, they found that eIF5A interacts with both p53 and syntenin, and over-expression of eIF5A resulted in an increase in p53, p21 and Bax, leading to p53-dependent apoptosis or sensitization of cells to undergo apoptosis induced by chemotherapeutic agents (Li et al., 2004). By contrast, down-regulation of eIF5A using RNAi resulted in reduced p53 levels (Li et al., 2004). Also, a point mutation of eIF5A that abolished hypusination did not result in p53 up-regulation, suggesting that hypusination, and thus DHS

activity, is necessary for p53-dependent apoptosis induction (Li et al., 2004). In sum, these results suggest that DHS activity and hypusination are necessary for apoptosis.

Similarly, Lee et al. (2002) found supporting evidence for the role of DHS, and thus hypusinated eIF5A, in apoptosis. They found that when they pre-treated human umbilical vein endothelial (HUVEC) cells with GC7 for four days at concentrations of up to 50 μM, the treatment seemed to elicit a protective effect against apoptotic cell death induced by serum starvation (Lee et al., 2002). Lee et al. (2002) confirmed that GC7 treatment of HUVEC cells resulted in a dose-dependent inhibition of hypusine formation and cell proliferation in the dose range of 5-50 μM over 2-5 days. They also observed significantly reduced cell viability after 5 days with GC7 treatment concentrations between 50-150 μM, and they concluded that this effect was probably due to toxic effects of GC7 at these high concentrations (Lee et al., 2002). In conclusion, evidence has been found for both pro- and anti-apoptotic roles of DHS and L-eIF5A, and further research will be needed to reconcile these seemingly contradictory research results.

1.5 Metastasis and deoxyhypusine synthase

The last step in neoplastic progression is metastasis, or the spreading of cancer cells from their original site to other sites in the body (Alberts et al., 2002.) Metastasis is the cause of 90% of deaths from solid tumors (Gupta and Massague, 2006). Metastasis progresses through a series of steps that are common in different types of cancers and, as a process, are often referred to as the metastatic cascade (Alberts et al., 2002; Kufe et al., 2003). In the case of solid tumors, the tumor is considered malignant, the condition typically called cancer, from the first step in the metastatic cascade (Alberts et al., 2002).

DHS seems to play an important role in metastasis as well. Ramaswamy et al. (2003) compared the gene-expression profiles of human adenocarcinoma metastases of various types of tumors and unmatched primary adenocarcinomas. They found a subset of tumors in the primary tumors that had similar gene-expression profiles as the metastatic tumors, and thus, they concluded that the results support the hypothesis that metastatic potential is encoded in the bulk of the primary tumor and does not arise from rare cells that have the ability to metastasize within the tumor (Ramaswamy et al., 2003). *dhs* was identified as one of eight signature metastatic genes, as these genes were found to be significantly up-regulated in metastases compared to non-metastatic tumors (Ramaswamy et al*.,* 2003). Four out of the eight up-regulated genes are components of the protein translation apparatus (DHS, SNRPF (small nuclear ribonucleoprotein F), EIF4EL3 (elongation initiation factor 4E-like 3), HNRPAB (Heterogeneous nuclear ribonucleoprotein A/B)) (Ramaswamy et al., 2003). Furthermore, over-expression of translation factors has been associated with tumor growth and invasion (Anand et al., 2002). Thus, *dhs* up-regulation seems to be important in metastasis.

DHS is likely to play a role in metastasis through its catalytic role in the hypusination of eIF5A2. eIF5A-2 over-expression has been correlated positively with colorectal carcinoma lymph node and distant metastasis stages of tumor development (Xie et al., 2008). Xie et al. (2008) concluded that eIF5A2 over-expression in colorectal carcinomas may be important in acquiring a metastatic phenotype as well as in cancer development and progression. In addition, significant association was found between eIF5A2 over-expression and tumor stage in ovarian cancer (Guan et al., 2004). Similarly, eIF5A2 was found to be one of three genes that could

correctly predict lymph node metastasis in 95% of cases of gastric cancer (Marchet et al., 2006). Thus, eIF5A studies also suggest that DHS is important for the metastatic ability of cancer cells.

1.6 Objectives and hypotheses

The goal of the present research was to investigate the role of DHS in invasiveness, cell proliferation and apoptosis in cancer cells using RNA interference. Based on previous research, DHS was expected to be involved in invasiveness, L-eIF5A was expected to be involved in apoptosis, and H-eIF5A was expected to be involved in cell proliferation. Consequently, the following specific hypotheses formed the basis of the research: (1) down-regulation of DHS with RNAi decreases cell invasiveness, (2) down-regulation of DHS with RNAi decreases cell proliferation, and (3) down-regulation of DHS with RNAi increases apoptosis.

Chapter 2
Materials and Methods

2.1 Cell culture

Three human cancer cell lines were used as experimental and control systems: (1) PC3, (2) HT-29, and (3) HeLa. PC3 is a human prostate adenocarcinoma cell line initiated from a bone metastasis (Kaighn, 1979). PC3 cells have been found to express eIF5A1 at the protein level and to over-express DHS at the mRNA level, but their eIF5A2 expression is very low (Clement et al., 2006). Thus, PC3 cells were used in the experiments because of their metastatic phenotype and their over-expression of DHS. PC3 cells were a gift from Anita Antes, University of Medicine and Dentistry of New Jersey, Newark, New Jersey, USA. PC3 cells were maintained in RPMI-1640 (Sigma) medium supplemented with 10% Fetal Bovine Serum (FBS) (Sigma).

HT-29 is a human colorectal adenocarcinoma cell line originally developed by Fogh and Trempe (1975). It has been used in a large number of published experiments and has been used successfully in the Thompson Lab for several eIF5A-related studies. The HT-29 cells were purchased from ATCC. HT-29 cells were maintained in RPMI-1640 medium supplemented with 10% FBS, supplemented with 10mM HEPES (Sigma), 1mM sodium pyruvate (Sigma), and 2.5 g/l sterile glucose (Sigma).

Finally, HeLa S3 is a human cervical adenocarcinoma cell line, a clonal derivative of the parental HeLa line (Puck et al., 1956). HeLa is also a very popular cell line for cell and molecular biology research. HeLa cells have also been used successfully for previous studies on DHS (Young et al., 1995; Klier et al., 1995). The HeLa S3 cells used in the present study were

also a gift from Anita Antes, University of Medicine and Dentistry of New Jersey. HeLa cells were maintained in RPMI-1640 medium supplemented with 10% FBS supplemented with 0.1mM non-essential amino acids (Sigma) and 4 g/l sterile glucose (Sigma). All cell lines were maintained at 37 ˚C in a humidified incubator with 5% carbon dioxide.

2.2 Gene silencing with RNAi

RNA interference (RNAi) is a very popular, gene-specific silencing mechanism widely used in biological and medical research. RNAi is an endogenous gene silencing mechanism that can also be triggered by exogenous RNA strands. For specific gene silencing in mammalian cells, small interfering RNAs (siRNAs), approximately 19-22 nucleotide-long double-stranded RNA strands with 2-base 3' overhangs, are used successfully. Once taken up by cells, the double-stranded exogenous RNAs are separated, and one strand complexes with the RNA Induced Silencing Complex (RISC), which then guides the complex to base-pair with complementary mRNA sequences and degrades them (Meister and Tuschl, 2004). To target certain genes, siRNAs that base-pair to specific mRNAs only can be designed using various algorithms, which are based on several design rules, such as the "Tuschl rules" (Ambion, 2006). The designed siRNAs can be ordered to be chemically synthesized by various commercial companies such as Dharmacon, Inc.

2.2.1 DHS siRNA design

For the siRNA design, review papers on the effectiveness of various available algorithms were studied. In addition, published papers on some of the most highly ranked algorithms were examined. The algorithms reported to be best able to predict siRNA effectiveness of experimentally tested siRNAs were selected for use in this study (Yiu et al., 2005; Ui-Tei et al.,

2006; Ding et al., 2004; Chalk et al., 2004). The selected list also included the algorithm that had been used successfully previously in the Thompson Laboratory for siRNA design. The algorithms used were as follows: Block-it (Invitrogen) (http://www.dharmacon.com/DesignCenter/DesignCenterPage.aspx), S-fold/Sirna (http://sfold.wadsworth.org/index.pl) (Ding et al., 2004), siSearch (Karolinska Institute, http://sisearch.cgb.ki.se/), and siDESIGN Center (Dharmacon) (http://www.dharmacon.com/DesignCenter/DesignCenterPage.aspx). The potential short-listed siRNAs were tested using BLAST (Basic Local Alignment Search Tool) to ensure specificity; that is, to ensure that they only base-pair to their targeted DHS mRNAs. Each algorithm ranked the siRNAs it recommended using various ranking systems including a numerical and a star system. A short list of about 15 siRNAs was created based on the best siRNAs predicted by the algorithms. Subsequently, the potential siRNA sequences were tested for immunostimulatory and cytotoxicity effects. For instance, a 4-base-pair motif "UGGC" in the RISC-entering strand of the siRNA is known to cause immunostimulatory effects (Fedorov et al., 2006); whereas, the 5'-GUCCUUCAA-3' motif in either strand has been found to cause cytotoxicity (Hornung et al., 2005). In addition, the potential siRNA sequences were tested for off-target effects caused by one or more perfect 3' UTR (untranslated region) matches with the hexamer or heptamer seed region in positions 2-7 or 2-8 in the antisense strand of the siRNA (Birmingham et al., 2006). The five best siRNAs, based on several algorithm rankings, were ordered and further tested experimentally (Table 1).

In addition, for transfection efficiency testing, a highly effective siRNA was used in both HT-29 and PC3 cells. The selected siRNA "positive control" siRNA had been successfully used

in the Thompson Laboratory to transfect HT-29 cells using the transfection protocol used in the current study (Taylor et al., 2007). The siRNA, which targets the 3' UTR of eIF5A-1 mRNA, was designed by Catherine Taylor in the Thompson Laboratory, and its sequences are as follows: sense strand 5'-GCUGGACUCCUCCUACACAdTdT-3' antisense strand 5'-UGUGUAGGAGGAGUCCAGCdTdT-3'.

Two non-targeting siRNAs were used in the current study. Control1 scrambled siRNA was designed by Catherine Taylor in the Thompson Lab. Control1 siRNA has a sense strand 5'-ACACAUCCUCCUCAGGUCGdTdT-3' and antisense strand 5'-CGACCUGAGGAGGAUGUGUdtdt-3'. Control 2 siRNA was a non-targeting control siRNA from Dharmacon. This non-targeting control siRNA was microarray tested to ensure that no human mRNA is targeted with its sequence. The control siRNA (Ctrl2 siRNA) has a sense strand 5'-UAGCGACUAAACACAUCAAUU-3' and the antisense strand 5'-UUGAUGUGUUUAGUCGCUAUU-3'.

2.2.2 siRNA transfection with Lipofectamine 2000

To facilitate siRNA uptake by mammalian cells, Lipofectamine 2000, a cationic lipid-based transfection reagent was used (Invitrogen). Lipofectamine is a widely-used, easy to use, and very effective transfection reagent that has minimal cytotoxicity. It works well with a wide variety of cell lines, including PC3.

Specifically, PC3 cells were seeded in 24-well plates at 35,000 cell density one day before transfection. On the day of the transfection, cells were placed in serum-free medium (RPMI-1640) and were transfected using siRNAs, Lipofectamine 2000, and Optimem, a reduced

serum medium (Gibco), with a final siRNA concentration of 0.14 μM. Cells were incubated with the transfection mix for 4 hours in a humidified incubator at 37 ˚C, 5% CO_2. After 4 hours, 150 μl RPMI-1640 medium supplemented with 30% FBS was added to each well bringing the FBS concentration in the wells close to 8%. The medium was replaced with RPMI-1640 with 10% FBS the following day.

2.3 Apoptosis induction

In this study, apoptosis was induced using two different chemicals. Actinomycin D is a widely known apoptosis-inducing agent. It exerts its effect by binding to DNA and repressing DNA replication and transcription (Guy and Taylor, 1978). Actinomycin D was used at 0.5 μg/ml (w/v) concentration dissolved in dimethyl sulfoxide (DMSO). HT-29 cells were treated with Actinomycin D for 48 hours before analysis. The second apoptosis-inducing chemical used in this study was sodium nitroprusside (SNP). SNP generates reactive oxygen species and nitric oxide, iron, and cyanide (Ramakrishna and Cederbaum, 1996). SNP has been found to induce apoptosis in various cell lines at mM concentrations (Taylor et al., 2007). In the current study, SNP was used at 10 mM concentration. PC3 cells were treated with SNP for 24 hours before analysis.

2.4 Protein level analysis

2.4.1 Whole cell lysate preparation

2.4.1.1 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) analysis

Protein analysis was carried out on whole cell lysate from cultured cells. Whole cell lysates were obtained by washing cells with cold phosphate buffered saline (PBS) (Sigma) and

treating them with lysis buffer (2% SDS (w/v), 62.5 mM Tris-HCl pH 7.4, 10% glycerol, and protease inhibitor cocktail 1x) (100 µl – 200 µl, depending on cell density). Cell lysates were sonicated to shear DNA and cleared at 13000 rpm for 10 minutes at 4˚C. After centrifugation, supernatants were collected, transferred into new tubes and stored at - 20˚C until further analysis.

2.4.1.2 Two Dimensional (2D) SDS-PAGE analysis

For 2D SDS-PAGE, cells were treated in a different way. After removal of the cell culture medium, cells were washed with cold PBS and incubated with lysis buffer (7M urea, 2M thiourea, 30mM Tris, 4% (w/v) CHAPS, protease inhibitor cocktail (1x)) for 30 minutes on ice with gentle shaking. Subsequently, whole cell lysates were treated as described above in Section 2.3.1.1.

2.4.2 Protein quantitation

Total protein concentration in cell lysates was determined using various protein quantitation methods depending on the lysis buffer used.

2.4.2.1 Bicinchoninic Acid (BCA) method

The BCA method was used in many cases since it is a quick and highly accurate protein quantitation method. The BCA reagent changes color based on the amount of total protein concentration in the sample, and thus, allows for colorimetric detection with a microplate reader. Total protein concentration in the samples is determined by comparing absorbance readings to serially diluted bovine serum albumin (BSA) standards.

Specifically, 10 µl diluted samples (1: 5 dilution) were pipetted in triplicate into wells of 96-well plates. BSA protein standards (Sigma-Aldrich) were prepared in duplicate. The BCA

protein assay kit (Sigma-Aldrich) was used following the manufacturer's instructions. The plates were then incubated for 15 min at 55˚C. Absorbance was measured using a microplate reader (Versamax Tunable microplate reader (Molecular Devices)) at 562 nm with background correction. A standard curve was calculated based on the standard absorbance values, and sample protein concentrations were calculated using the equation of the standard curve.

2.4.2.2 Bradford method

In cases in which chemicals in the lysis buffer were incompatible with BCA quantitation, as in the lysis buffer for 2D-SDS-PAGE, the Bradford assay was used for protein quantitation. The BSA standards were the same as in the case of the BCA method.

Specifically, 100 µl of diluted cell lysate (1:5 dilution) was mixed with 1000 µl Bradford reagent 1x (Bio-Rad). Standards were prepared in duplicate. The absorbance values were read at 595 nm by a DU-64 spectrophotometer. A standard curve was calculated based on standard absorbance values, and sample protein concentrations were determined using the standard curve equation.

2.4.3 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

In order to detect and analyze various proteins of interest, the total protein content of the samples needed to be fractionated. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) is a technique that allows for separation of proteins based on molecular weight (Alberts et al., 2002). Specifically, for analysis of DHS and β-actin (loading control), 10% (w/v) resolving polyacrylamide gel was used with 5% (w/v) stacking gel. By contrast, for eIF5A-1 detection and β-actin (loading control), 12% (w/v) resolving polyacrylamide gel was used with

5% (w/v) stacking gel. In both cases, protein samples were diluted in loading buffer (320 mM Tris-HCl pH 6.8, 40% (v/v) glycerol, and 8% (w/v) SDS, 0.04% Bromophenol Blue, 20% β-Mercaptoethanol). The samples were boiled for 5 minutes at 100 ˚C. Samples were loaded into each lane so that the total loaded protein per lane was either 1 µg or 5 µg, with the same amount of protein per lane in each gel. In order to provide a reference migration distance on the gel, 7 µl of pre-stained protein ladder (Fermentas) was loaded into a lane. The gels were run in SDS PAGE running buffer (25mM Tris, 250 mM glycine, 0.1% (w/v) SDS) at 40 mA until the desired protein separation was achieved. With the stacking gel removed, the separating gel was placed in transfer buffer (25mM Tris-HCl pH 8.3, 192 mM glycine, 20% (v/v) methanol) for 5 minutes. Hybond-P Polyvinylidene Fluoride (PVDF) membrane (Amersham Biosciences) was pre-wetted with 100% (v/v) methanol for 5 minutes and transferred to transfer buffer. For protein transfer from the polyacrylamide gel to the PVDF membrane, a Trans-blot SD semi-dry transfer cell (Bio-Rad) was used according to the manufacturer's instructions. The gel was placed on the top of the membrane, and together they were placed on the transfer cell inserted between two pieces of blotting papers pre-wetted in transfer buffer. The transfer was run for 40 minutes at 20 mV.

2.4.4 Western blotting

Protein bands on the PVDF membranes need to be detected before they can be visualized. In order to detect the protein of interest, mono or polyclonal antibodies can be used. First, the membrane needs to be blocked with a blocking solution to prevent non-specific antibody binding, then the membrane is incubated with primary antibodies which bind the protein of interest. After extensive washing, the membrane is incubated with the appropriate secondary

antibody that binds the primary antibody. Usually the secondary antibody is conjugated to an enzyme, for instance horseradish peroxidase, which aids in visualization (Alberts et al., 2002).

Specifically, to detect DHS, affinity purified polyclonal antibody (IgG) against DHS raised in goat was obtained from Santa Cruz Biotechnology, Inc (Santa Cruz, CA) in two versions: (1) raised against a peptide mapping near the N-terminus of human DHS protein, and (2) raised against a peptide mapping near the C-terminus of human DHS protein. The antibodies were diluted in blocking solution 1:800 in 1% bovine serum albumin (BSA). The secondary antibodies used were donkey anti-goat IgG-horse radish peroxidase (HRP) from Santa Cruz Biotechnology, Inc diluted in blocking solution 1:10,000 in 1% BSA. For loading controls, mouse anti β-actin (42KDa) antibodies (IgM) were used at a dilution of 1:20,000 in 5% milk, and rabbit anti-mouse antibodies (IgM) conjugated to HRP were used at 1:20,000 dilution in 5% milk. Parallel blots were run with the same amount of loaded total protein and detected with anti-β-actin antibodies, or following the detection of DHS, the membranes were stripped and re-probed with anti-β-actin antibodies. For eIF5A-1 immunodetection, the primary antibodies were mouse anti-eIF5A-1 IgG antibodies in 5% milk (1:10,000), while the secondary antibodies were anti-mouse IgG conjugated to HRP in 5% milk (1:20,000).

2.4.5 Detection

In order to visualize the protein of interest with the antibodies bound to it, chemiluminescence was used. Specifically, chemiluminescence entails using a chemical substrate that the enzyme conjugated to the secondary antibody can act on and can produce a detectable signal. Specifically, ECL Plus (Enhanced Chemiluminescence Plus) Western blotting detection reagent (Amersham) was used following the manufacturer's instructions. 2.5 ml of

mixed detection solution (reagents A and B mixed in a ratio of 40:1) was used per 6 cm by 8 cm membrane. Membranes were incubated for three to five minutes, depending on signal strength. Subsequently, the signal was detected using X-ray film with an AGFA CP 1000 X-ray film processor (AGFA).

2.4.6 Stripping and repeated Western blotting

In order to redetect the same membrane with different antibodies, the membrane can be stripped with a stripping buffer. Specifically, the membranes were incubated in a stripping buffer (100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl pH 6.7) for 15 – 20 minutes at 55 ˚C with slight agitation. The membranes were washed three times with PBS-T (Phosphate buffered saline with 0.05 % Tween 20 (v/v)) for 10 minutes with gentle shaking. The membranes were then blocked again with the blocking solutions and re-probed with new antibodies.

2.4.7 Two-dimensional gel electrophoresis

In order to separate the various post-translationally modified forms of eIF5A, that is L-eIF5A and H-eIF5A, two-dimensional Gel Electrophoresis (2D SDS-PAGE) was used. 2D SDS-PAGE combines two separation methods. First, proteins are separated by their intrinsic charges. This first step is called isoelectric focusing, and proteins migrate in an electric field in a pH gradient to the point where they have no net charge; that is, they are at their isoelectric point (Alberts et al., 2002). Second, proteins are subjected to a regular SDS-PAGE, and consequently, proteins are separated according to their size. Subsequently, the SDS-PAGE gel is treated as described for the one-dimensional PAGE above.

In detail, isoelectric focusing was performed using an Ettan IPGphor Isoelectric Focusing System (Amersham) according to the manufacturer's instructions. For eIF5A, 7 cm, DryStrips (pH 4-7) from Amersham were used. The strips were rehydrated for 12 hours at 25 ˚C in a rehydration buffer (8M urea, 2% (w/v) CHAPS, 0.2% (w/v) DTT, 0.5% (v/v) pH 4-7 IPG buffer and 0.002% (v/v) bromophenol blue) with 3 μg or 5 μg total protein. This step was followed by isoelectric focusing at 500V for 30 min; 1000V for 10 min; and 2500V for 100 min. Subsequently, the protein in the IPG (Immobilized pH Gradient) strip was equilibrated in SDS equilibration buffer (50mM Tris-HCl pH 8.8, 6M urea, 30% (v/v) glycerol, 2% (w/v) SDS and 0.002% (w/v) bromophenol blue) with 10 mg/ml dithiothreitol (DTT) (Fermentas) for 15 minutes at room temperature on a shaker. Next, the strips were incubated in equilibration buffer with 25 mg/ml iodoacetamide (IAA) (Sigma) for another 15 minutes under similar conditions. Finally, one strip per SDS-PAGE gel was placed on the top of a 12% separating gel and sealed with agarose sealing solution (0.5% (w/v) agarose and 0.002% (w/v) bromophenol blue, 25mM Tris, 250mM glycine, 0.1% SDS). Proteins were separated, detected, and visualized following the same steps as described above in Sections 2.4.3 - 2.4.5.

2.5 Cell invasion

2.5.1 Matrigel invasion assay

A widely used *in vitro* assay for assessing the metastatic potential of tumor cells is the Matrigel Matrix assay (BD Biosciences). Matrigel Matrix is a biologically active reconstituted extracellular matrix preparation derived from the Englebreth-Holm-Swarm (EHS) mouse tumor which is rich in extracellular matrix proteins. The major components of the Matrigel Matrix are laminin, collagen IV, heparan sulfate proteoglycans, entactin, and nidogen. Matrigel Invasion

Chambers are Boyden-like chambers with PET (polyethylene terepthlate) membrane with 8 micron pores coated with a thin layer of Matrigel Matrix (BD Biosciences). The membrane serves as a reconstituted basement membrane *in vitro*. The thin Matrigel membrane prevents the migration of non-invasive cells, but invasive cells are able to detach from and invade the Matrigel membrane, and they can be visualized using a cell stain and counted with a microscope. Cell invasion through Matrigel is aided by chemoattractants typically found in FBS. Matrigel Invasion assays were performed using BD Matrigel Invasion Chambers (BD Biosciences) according to the manufacturer's instructions.

Specifically, Matrigel Invasion Chambers/Transwells were rehydrated for 2 hours at 37˚C in serum-free medium (RPMI-1640). PC3 cells were trypsinized and suspended in 0.1 % FBS supplemented RMPI-1640 medium, centrifuged at 1000 rpm, re-suspended in serum-free medium, counted, and diluted to a 100,000 cells/ml concentration. The rehydration medium was removed from the inserts, and the inserts were placed in 500 µl medium with 10 % FBS per well. 500 µl of cell suspension was placed on the top of the inserts, and they were incubated for 48 hours at 37˚C. Subsequently, the cell suspension and the remaining Matrigel layer were removed from the insert, the invaded cells were stained with Cell Stain from a Cell Invasion Kit (Chemicon International), and the cells on the lower surface of the membrane were counted under a microscope.

2.6 Cell proliferation measurement

2.6.1 Cell counting using Trypan blue

A simple method of measuring cell proliferation in cell culture is to count viable cells using the Trypan blue. The technique is based on the disruption of the cell membrane that distinguishes non-viable and viable cells. The disrupted membrane of non-viable cells allows the Trypan blue dye to be taken up by the cell, and thus viable cells can be visualized under microscope as colorless cells compared to the blue-colored non-viable cells. Viable cells can be counted repeatedly over a time course, and thus, the rate of cell proliferation can be determined. Specifically, ten µl of Trypan blue (concentration 0.4% w/v) was added to ten µl cell suspension. After mixing and an incubation time of 2-3 minutes, the cell-stain mixture was loaded into a hemocytometer, the colorless cells were counted.

2.6.2 XTT cell proliferation assay

An alternative quick and easy method of measuring cell proliferation is the XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide) assay. This assay is based on a color change in a dye produced by viable—metabolically active—cells; thus, it is a cell viability assay used to measure cell proliferation. XTT is a tetrazolium salt that is metabolically reduced in viable cells to an orange-colored, water soluble formazan product that can be measured using a microplate reader (Scudiero *et al.,* 1988). XTT is a widely used method of measuring cell proliferation and cell viability.

Specifically, cells were seeded on 96-well plates after transfection, and at various times 50 µl XTT reagent and activating solution mix (mixed 50:1) was added to each well. The plates were then incubated at 37˚C for 2 hours. Alternatively, cells were seeded on 96-well plates at

5000 cells per well, transfected, and XTT assays performed at various time points. Absorbance values were obtained using a Versamax Tunable microplate reader (Molecular Devices) at 490 nm and 630 nm (background) wavelengths.

2.7 Apoptosis measurement

2.7.1 Annexin V/PI apoptosis assay

Apoptosis was measured using the Annexin V/PI apoptosis assay and a Fluorescence Activated Cell Sorter (FACS). This technique is based in part on changes in the plasma membrane that occur during apoptosis. Early in apoptosis phosphatidylserine, which in viable cells can only be found on the inner-membrane leaflet, is translocated to the outer leaflet, and thus allows for detection of apoptosis (van Engeland *et al.,* 1998). Annexin V, a phospholipid-binding protein, interacts strongly and specifically with phosphatidylserine. Annexin V is labeled with a commonly used fluorecent dye, FITC (excitation wavelength 488nm; emission wavelength 525nm), which allows its detection using fluorescence activated - cell sorting (FACS). Usually a vital dye, Propidium Iodide (PI) is used with Annexin V to stain non-viable cells. Thus, viable cells are typically Annexin V-negative and PI-negative, early apoptotic cells are Annexin V-positive but PI-negative, and finally, late apoptotic cells or dead cells are Annexin V-positive and PI-positive. Annexin V-FITC /PI assay results are often presented in dot blots where the x axis represents Annexin V-FITC labeling and the y axis represents PI labeling. Consequently, cells in the lower left quadrant of the histograms are viable cells, Annexin V-FITC-negative and PI-negative; cells in the lower right quadrant are early apoptotic cells, Annexin V-FITC-positive and PI-negative; and cells in the upper right quadrant are late apoptotic or dead cells, Annexin V-FITC-positive and PI-positive. The assay was prepared using

the Annexin V-FITC apoptosis detection kit I (BD Bioscience) according to the manufacturer's instructions. The following controls were used for the Annexin V-FITC /PI assay: untreated and unstained cells, untreated cells treated with Annexin V only, and untreated cells treated with PI only. For a positive control for apoptosis, cells were treated with Actinomycin D, or SNP, stained with both Annexin V-FITC and PI, and stained individually, only with Annexin V-FITC or only with PI.

Specifically, cells were seeded in 24-well plates 35,000 cells per well, and they were transfected with DHS 0 siRNAs, DHS 1/b siRNAs, treated with Actinomycin D, or with SNP, or left untreated. For the apoptosis assay, cells were trypsinized, washed, and stained with Annexin V-FITC and/or PI according to the manufacturer's instructions (BD Biosciences). Subsequently, the cells were analyzed by FACS (Coulter Epics XL-MCL) and the data was analyzed by WinMDI 2.8 software.

Chapter 3
Results

3.1 Confirmation of a high expression level of DHS in PC3 cells

DHS has been shown to be over-expressed at the mRNA-level in PC3 cells (Clement et al., 2006). To confirm that DHS was expressed at a high level in PC3 cells at the protein level as well, PC3 cell lysate was fractionated on 10% SDS-PAGE separating gel, transferred to PVDF membrane, and the DHS protein was detected using anti-DHS antibodies raised in goat (1:800). The secondary antibodies were anti-goat antibodies raised in donkey conjugated to horseradish peroxidase (1: 10,000). For loading controls, mouse anti β-actin (42KDa) antibodies (IgM) were used as primary antibodies (1:20,000), followed by rabbit anti-mouse antibodies (IgM) conjugated to HRP as secondary antibodies (1:20,000). The antibodies were visualized using chemiluminescence. The resultant bands were analyzed using densitometry analysis with background correction. The actin normalized intensity of the DHS band for PC3 cells was 0.322, whereas the actin normalized intensity for HeLa cells was 0.011 (Figure 5). Thus, the results indicate that the DHS protein expression level in PC3 cells is approximately thirty times higher than the expression level in HeLa cells (Figure 5).

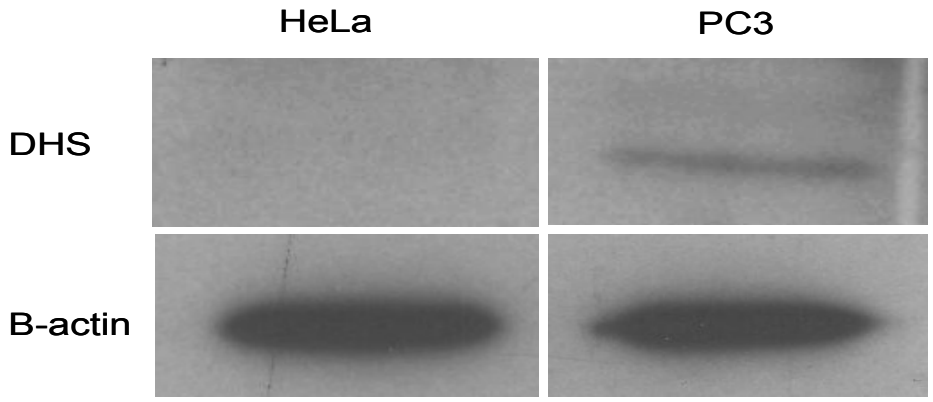
3.2 siRNA design against DHS mRNA

One objective of the study was to design siRNAs against DHS. Although one siRNA against DHS, DHS 0 siRNA, had already been used in the lab, it was felt desirable to have at least one additional DHS siRNA. DHS 0, a previously published DHS siRNA (Hauber et al.,

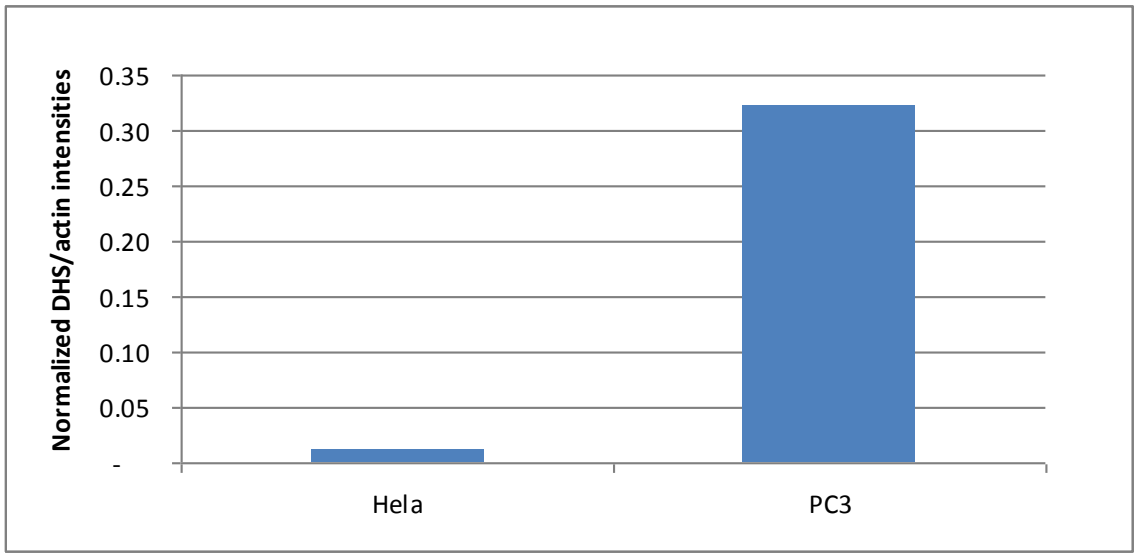
Figure 5. Comparison of DHS expression in PC3 and HeLa cells.

Panel A. Western Blots of HeLa and PC3 cell lysates detected with N-terminus anti-DHS primary antibodies. β-actin served as loading control. Panel B. Densitometry analysis of DHS protein expression levels in HeLa and PC3 cells normalized to β-actin loading controls. The densitometry analysis indicates that the DHS expression level in PC3 cells is approximately thirty times the DHS expression level in HeLa cells.

Panel A.



Panel B.



2005), served as a benchmark and has been classified as "a medium effectiveness" siRNA in siRecords, a database of mammalian siRNAs that includes effectiveness ratings (Ren et al., 2006). Various algorithms were used to choose the most promising additional siRNAs, including Dharmacon's siDESIGN, which classifies recommended siRNAs with a rank number (http://www.dharmacon.com/DesignCenter/DesignCenterPage.aspx) , Sfold (http://sfold.wadsworth.org/index.pl) (Ding et al., 2004), siSearch (Karolinska Institute, http://sisearch.cgb.ki.se/) (Chalk et al., 2004), which also uses a rank number, and Block-it (Invitrogen), which provides star ratings of the recommended siRNAs (http://www.dharmacon.com/DesignCenter/DesignCenterPage.aspx). DHS1/a siRNAs and DHS 1/b siRNAs differ only in their overhangs: DHS1/a siRNAs have dTdT overhangs, whereas DHS 1/b siRNAs have dAdC overhangs. Cytotoxicity and immunostimulatory effects were evaluated based on known sequence motifs causing cytotoxicity or immunostimulatory effects (Fedorov et al., 2006; Hornung et al., 2005). All newly designed siRNAs, except DHS2 siRNAs, target all three transcript variants of the human DHS gene (Table 1).

3.3 siRNA effectiveness evaluation

The effectiveness of the various DHS siRNAs was evaluated at two levels: at the protein level and at the enzyme activity level. First, the appropriateness of the transfection protocol in PC3 cells was confirmed. Second, the ability of the various DHS siRNAs to decrease DHS protein levels was evaluated using SDS-PAGE and Western blotting. Third, the ability of the various DHS siRNAs to inhibit DHS enzyme activity was assessed using two-dimensional Gel Electrophoresis (2D SDS-PAGE).

Table 1. Summary of the sequences and features of siRNAs ranked by various algorithms.

The benchmark was the DHS 0 siRNA, a previously published DHS siRNA (Hauber et al., 2005), which was classified as "a medium effectiveness" siRNA in siRecords, a database of mammalian siRNAs with effectiveness ratings (Ren et al., 2006). Various algorithms were used to choose the most promising siRNAs, including Dharmacon's siDESIGN, which classifies recommended siRNAs with a rank number, Sfold (Ding et al., 2004), siSearch (Karolinska Institute), which also uses a rank number, and Block-it (Invitrogen), which provides star ratings of the recommended siRNAs. DHS1/a siRNAs and DHS 1/b siRNAs differ only in their overhangs: DHS1/a siRNAs have dTdT overhangs, whereas DHS 1/b siRNAs have dAdC overhangs. Cytotoxicity and immunostimulatory effects were evaluated based on known sequence motifs causing cytotoxicity or immunostimulatory effects (Fedorov et al., 2006; Hornung et al., 2005). All newly designed siRNAs, except DHS2 siRNAs, target all three transcript variants of the human DHS gene.

siRNA Name	Sense Sequence (5'->3')	Antisense Sequence (5'->3')	Starting Nucleotide	Algorithm/ Source	Major Features
DHS 0	CAUGGUGGACGUAUUGGUG	CACCAAUACGUCCACCAUG	367	Hauber et al., 2005	Medium effectiveness by siRecords (Ren et al., 2006) Overhangs: dTdT
DHS 1/a	GUAAAGUGGACGCCUUCUA	UAGAAGGCGUCCACUUUAC	692	Best of Dharmacon (8/10), Sfold	All 3 transcript variants No immunostimulatory effect No cytotoxicity effect Overhangs: dTdT
DHS 1/b	GUAAAGUGGACGCCUUCUA	UAGAAGGCGUCCACUUUAC	692	Best of Dharmacon (8/10), Sfold	All 3 transcript variants No immunostimulatory effect No cytotoxicity effect Overhangs: dAdC
DHS 2	CCGACUACGCUGUUUACAU	AUGUAAACAGCGUAGUCGG	996	Block it - Invitrogen 5 stars, Sfold	Transcript variant 1only No immunostimulatory effect Overhangs: dTdT No cytotoxicity effect
DHS 3	GGGAUCAAUAGGAUCGGAA	UUCCGAUCCUAUUGAUCCC	581	Block it - Invitrogen 5 stars	All 3 transcript variants No immunostimulatory effect Overhangs: dTdT No cytotoxicity effect
DHS 4	CACUGUCACAGGAUGAAGA	UCUUCAUCCUGUGACAGUG	324	Karolinska – (7/10)	All 3 transcript variants No immunostimulatory effect Overhangs: dTdT No cytotoxicity effect

3.3.1 Confirmation of the transfection efficiency in PC3 cells

Transfection efficiency of the transfection protocol was confirmed in PC3 cells using a high efficacy siRNA, eIF5A-1 siRNA, which had been found to knock-down the eIF5A-1 protein in HT-29 cells very effectively (Taylor et al., 2007). HT-29 cells and PC3 cells were transfected with eIF5A-1 siRNAs, Control1 siRNAs, or treated with Lipofectamine 2000 alone (Mock) (Figure 6). Cell lysates were collected 72 hours after transfection, fractionated on 12% SDS-polyacrylamide gel, and transferred to PVDF membrane. The primary antibodies used in the immunodetection were anti-eIF5A-1 mouse IgG antibodies in 5% milk (1:10,000), followed by anti-β-actin IgM in 5% milk (1:20,000). The antibodies were visualized using chemiluminescence. The resulting Western blots indicated that the eIF5A-1 siRNA treatment decreased eIF5A1 protein levels in PC3 cells comparably to the eIF5A suppression in HT-29 cells, suggesting that the transfection protocol used was appropriate for the transfection experiments in PC3 cells.

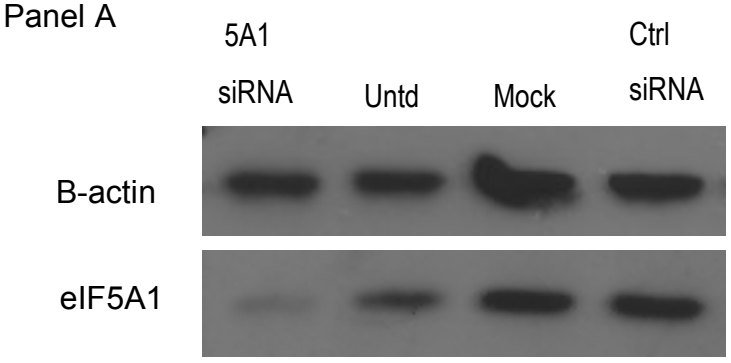
3.3.2 siRNA effectiveness evaluation at the protein level

In order to assess the effectiveness of the various DHS siRNAs to decrease DHS protein levels in PC3 cells, SDS-PAGE and Western blotting was used. PC3 cells were treated with the various siRNAs, and cell lysates were collected 72 hours after transfection and analyzed using SDS-PAGE with a 10% separating gel. The DHS protein was detected with N-terminus anti-DHS goat antibodies (1:800) in 1% bovine serum albumin (BSA) with secondary antibodies against goat (1:10,000) in 1% BSA. The loading controls were mouse anti-β-actin antibodies (1:20,000) in 5% milk and rabbit anti-mouse IgM-HRP in 5% milk (1:20,000).

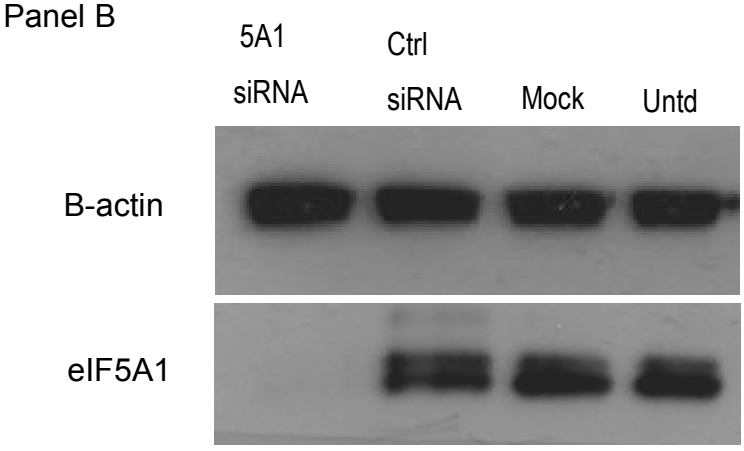
Figure 6. Confirmation of the transfection efficiency in PC3 cells.

The transfection efficiency of the transfection protocol in PC3 cells was confirmed using a very effective eIF5A-1 siRNA that had been found to deplete the eIF5A-1 protein in HT-29 cells very effectively (Taylor et al., 2007). HT-29 cells and PC3 cells were transfected with eIF5A-1 siRNAs, control siRNAs, or treated with Lipofectamine 2000 alone (Mock). Cell lysates were collected 72 hours after transfection, fractionated on 12% SDS-polyacrylamide gel, and transferred to a PVDF membrane. The primary antibodies used in the immunodetection were mouse anti-eIF5A-1 IgG antibodies in 5% milk (1:10,000), followed by anti-β-actin IgM in 5% milk (1:20,000). Panel A. Western blots of eIF5A1 siRNA, control siRNA, Lipofectamine 2000 alone (Mock) treated and untreated HT-29 cells showing eIF5A1 protein levels. β-actin was used as a loading control. Panel B. Western blots of eIF5A1 siRNA, control siRNA, Lipofectamine 2000 alone (Mock) treated and untreated PC3 cells showing eIF5A1 protein levels. β-actin was used as a loading control.

Panel A



Panel B



The antibodies were visualized using chemiluminescence. Densitometry values for the various DHS siRNAs were normalized to the respective β-actin loading controls. Background correction was included. The normalized densitometry value for the benchmark DHS 0 siRNAs was 0.178, whereas the corresponding value for DHS1/a siRNAs was 0.153, and for DHS 1/b siRNAs was 0.075, and for DHS4 siRNA 0.107. The highest level of DHS protein depletion was observed in DHS 1/b siRNA-treated cells (Figure 7). The sequences, overhangs, and specific characteristics of the various siRNAs are presented in Table 1.

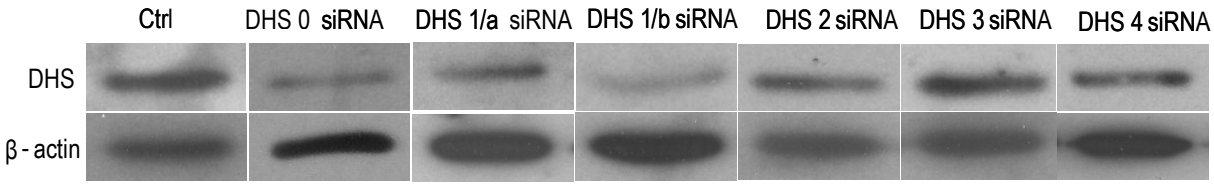
3.3.3 siRNA effectiveness evaluation at the enzyme activity level

DHS enzyme activity was measured in an indirect way. Since DHS catalyzes the first step of the hypusination pathway (Figure 1), inhibition of DHS activity either by decreasing DHS levels using DHS siRNAs or by competitive inhibition by using GC7, would result in the accumulation of L-eIF5A over time depending on the half life of DHS and eIF5A. Thus, the effectiveness of the inhibition of DHS activity was measured using 2D-SDS-PAGE and Western blotting with eIF5A1 primary antibodies and secondary antibodies conjugated to horseradish peroxidase which allowed visualization of the various forms of eIF5A (Figure 8). The first dimension, isoelectric focusing, allowed the separation of the various forms of eIF5A based on their different isoelectric points (pI). L-eIF5A, D-eIF5A, and H-eIF5A have isoelectric points of 4.9, 5.0, and 5.1, respectively. The second dimension of the 2D-SDS-PAGE separated the proteins based on their size, and the resulting 2D blots show the various forms of eIF5A. The identities of spot C as the hypusinated form and spot A as the lysine form were confirmed using mass-spectrometry (Sun, 2007). The results indicate that DHS 0 siRNAs and DHS 1/b siRNAs were both effective in causing the accumulation of L-eIF5A (spot A in Figure 8) in 2D-SDS-

Figure 7. Comparison of DHS siRNA effectiveness.

PC3 cells were transfected with DHS 0 siRNAs, DHS 1/a siRNAs, DHS 1/b siRNAs, DHS 2 siRNAs, DHS 3 siRNAs, and DHS 4 siRNAs or were left untreated (Ctrl). Cell lysates were collected 72 hours after transfection and were fractionated on 10% SDS-PAGE separating gels, followed by a transfer to PVDF membranes and Western blotting. Panel A.Western Blots showing the efficacy of various DHS siRNAs. DHS was detected using anti-N-terminus DHS antibodies. β-actin loading controls are also presented. Panel B. Densitometry analysis of DHS protein levels in the presence of various DHS siRNAs normalized to β-actin loading controls. Ctrl represents untreated cells. Values are shown as means +/- standard error. Based on the results, DHS 1/b siRNA has the highest level of effectiveness in decreasing DHS expression in PC3 cells. The sequences, overhangs, and specific characteristics of the various siRNAs are presented in Table 1.

Panel A.



Panel B.

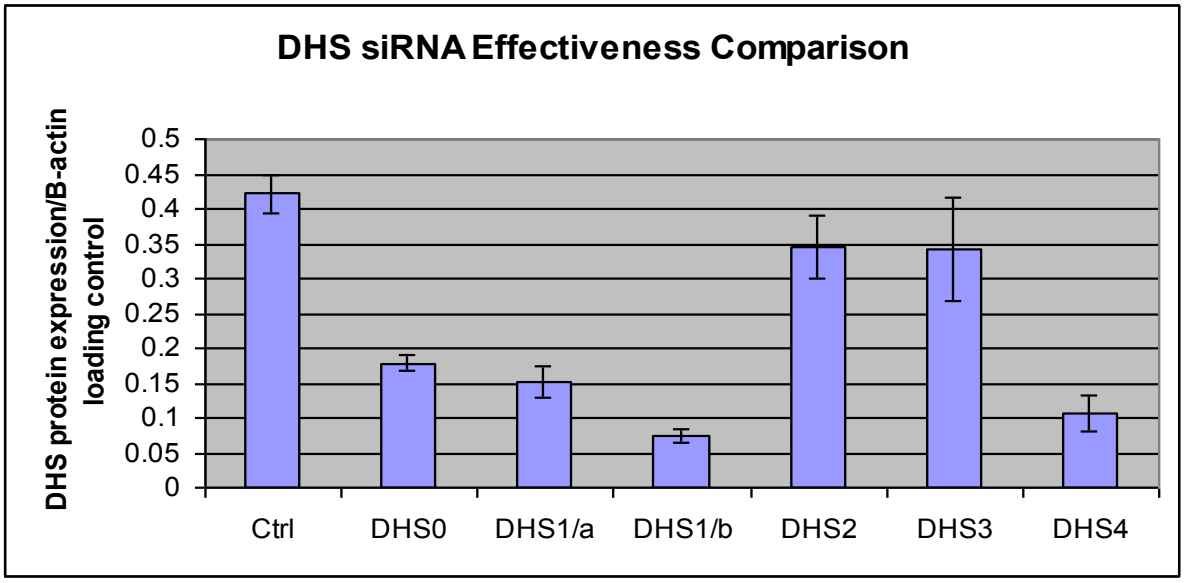
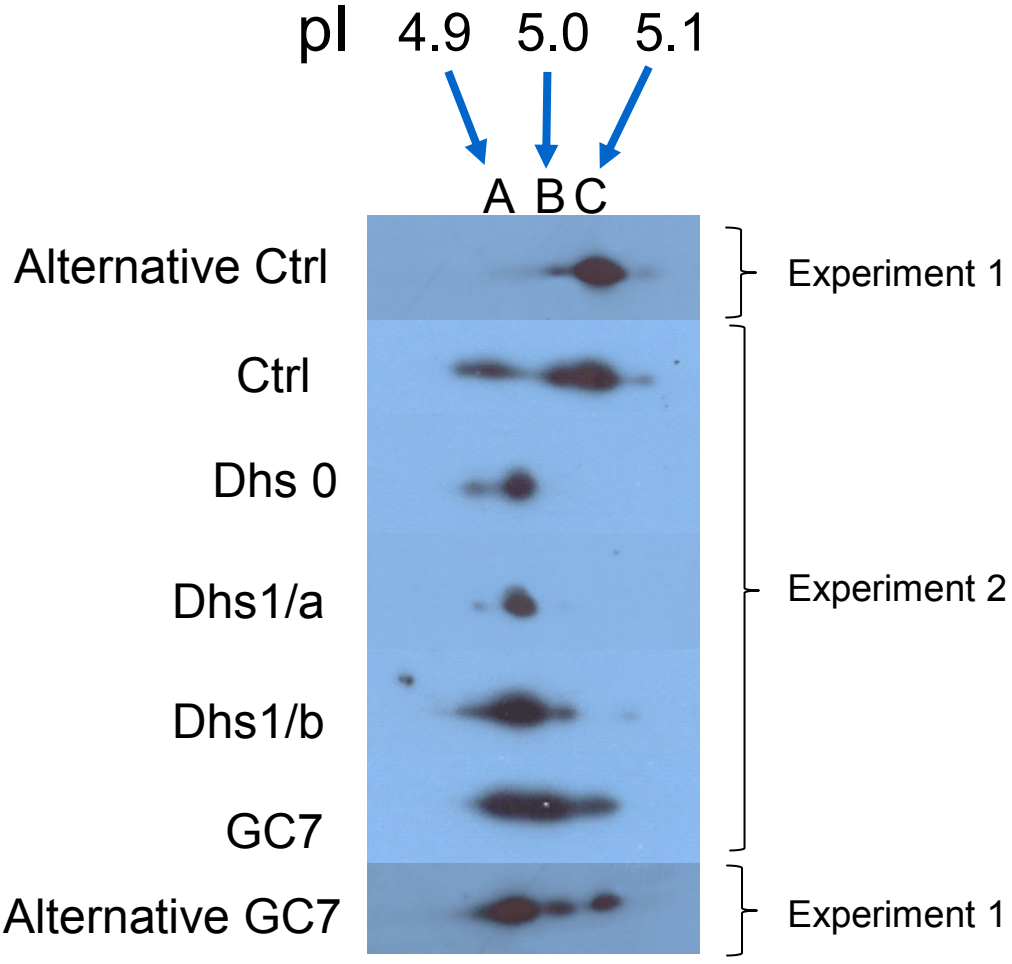


Figure 8. 2D gel electrophoresis and Western blot analysis of cell lysates from PC3 cells.

The first dimension of the 2D SDS-PAGE, isoelectric focusing, was performed using a 7cm long strip with a pH range of 4-7. The isoelectric points (pI) of the various forms of eIF5A are different, which allows their separation in the first dimension of the 2D SDS-PAGE. The pI values are 4.9 for the L-eIF5A, 5.0 for the D-eIF5A, and 5.1 for the H-eIF5A. The second dimension of the 2D SDS-PAGE is the SDS-PAGE followed by Western blotting. This phase was performed using anti-eIF5A1 primary antibodies. Spot A represents L-eIF5A, spot B represents D-eIF5A, and spot C represents H-eIF5A. Ctrl represents Ctrl siRNA-treated cells. Alternative Ctrl and alternative GC7 represent additional negative and positive controls which were run separately from a separate experiment. Based on the results, both DHS 0 siRNAs and DHS 1/b siRNAs are effective in increasing relative L-eIF5A levels and decreasing H-eIF5A levels, and thus decreasing DHS enzymatic activity.


pI 4.9 5.0 5.1
A B C
Alternative Ctrl
Ctrl
Dhs 0
Dhs1/a
Dhs1/b
GC7
Alternative GC7
Experiment 1
Experiment 2
Experiment 1

PAGE-Western blots. GC7-treated cells were used as positive controls, while the control siRNA-treated cells were used as negative controls. Two blots from different gel runs are presented in Figure 8. Based on the 2D SDS-PAGE results, both DHS 0 siRNAs and DHS 1/b siRNAs were effective in inhibiting DHS enzyme activity.

In sum, based on the SDS-PAGE and Western blotting results and the 2D SDS-PAGE and Western blotting results, DHS 1/b siRNAs were selected for further experiments. Since DHS1/a siRNAs only differ from DHS 1/b siRNAs in their overhangs, DHS1/a siRNAs were also included in certain experiments.

3.4 Invasiveness of PC3 cells in response to various siRNA treatments

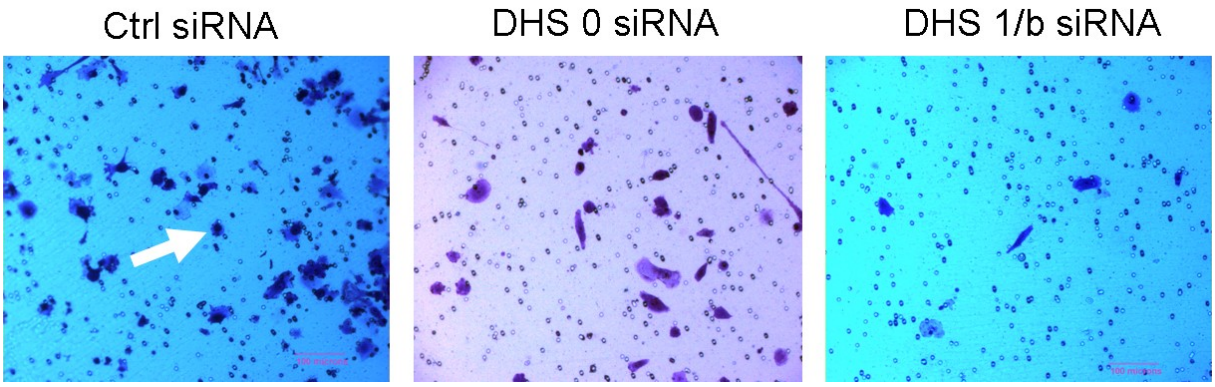
Invasiveness *in vitro* was measured using Matrigel-coated transwells. Cells were treated with DHS 0 siRNAs, DHS 1/b siRNAs, or non-targeting control siRNAs and were placed on the top of the Matrigel transwells in serum-free medium. The transwells were placed in full (10%) Fetal Bovine Serum-containing medium in wells of 24-well plates. After 48 hours, the Matrigel and the remaining cell suspension was removed from the transwells, and the cells that had migrated to the bottom of the polyethylene terephthalate (PET) membrane through the 8 μm pores were stained and counted under a microscope at 100x magnification. For each transwell, cells were counted in five fields, and the numbers of cells in the five fields were averaged (Figure 9). The experiment was repeated five times; however, DHS 0 siRNA-treatment was included in three experiments only.

The numbers of cells that invaded the Matrigel basement membrane and migrated to the bottom of the PET membrane were expressed as means +/- standard errors. Specific values are:

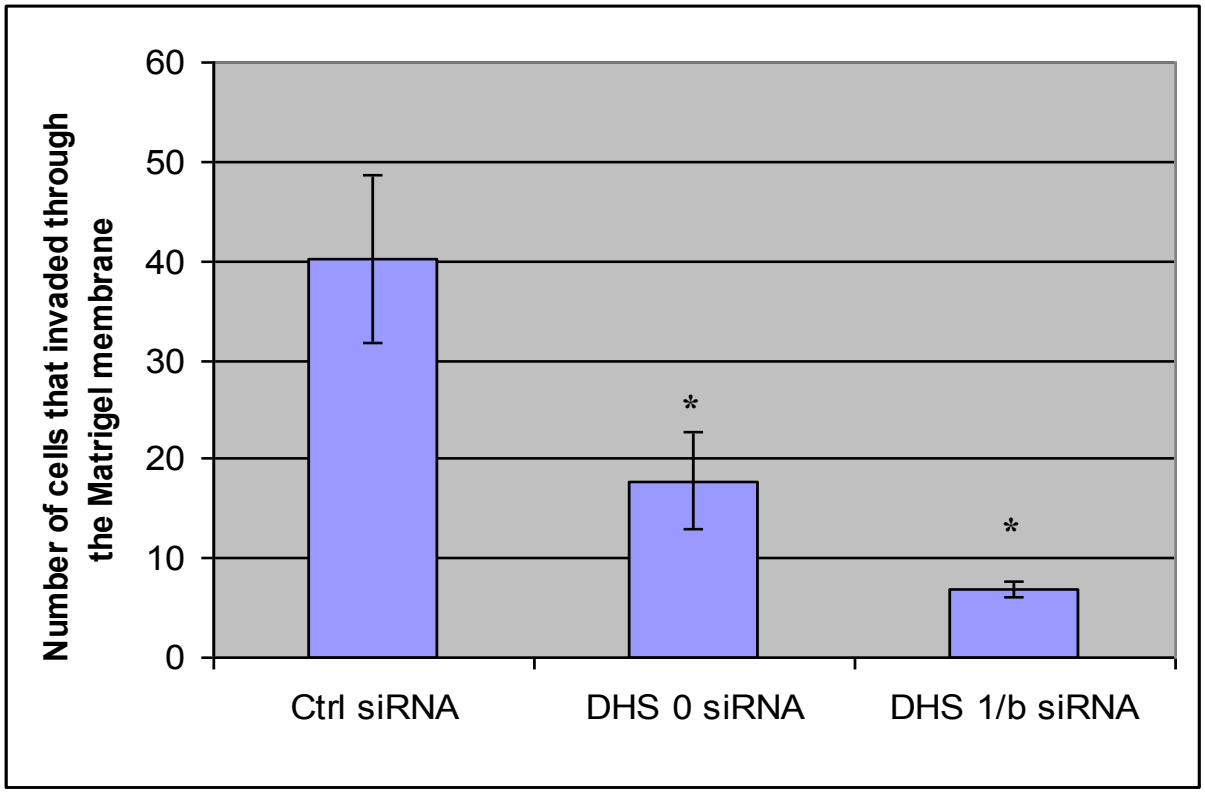
Figure 9. Invasiveness of PC3 cells through Matrigel basement membrane-coated transwells.

PC3 cells were treated with DHS 0 siRNAs, DHS 1/b siRNAs, or non-targeting control siRNAs for 48 hours. The treated cells were placed on the top of Matrigel invasion transwells, and after 48 hours the cells which had invaded the Matrigel membrane and migrated to the opposite side of the PET membrane were counted in five fields per invasion chamber. The experiments were run five times; however, DHS 0 siRNA treatment was included in three experiments only. Panel A. Images of cells that invaded through the Matrigel basement membrane. The arrow indicates a stained cell. Panel B. Bar graphs showing the number of cells that invaded through the Matrigel membrane. The results are expressed as number that invaded the Matrigel basement membrane and migrated through the PET membrane +/- standard error. The results were transformed to ln values and analyzed with one way ANOVA and Dunnett t as the post hoc test. Both the DHS 0 siRNA-treated cells and the DHS 1/b siRNA-treated cells were significantly less invasive than control siRNA treated cells (*$p<0.05$).

A


Ctrl siRNA
DHS 0 siRNA
DHS 1/b siRNA

B


Number of cells that invaded through the Matrigel membrane
60
50
40
30
20
10
0
*
*
Ctrl siRNA
DHS 0 siRNA
DHS 1/b siRNA

40 +/- 9, 18 +/- 5, and 7 +/- 1 for control siRNA-treated, DHS 0 siRNA-treated, and DHS 1/b siRNA-treated cells, respectively (Figure 9). For the purpose of the statistical analysis, the data were transformed to ln values. The results of the statistical analysis indicate that the invasiveness of both DHS 0- and DHS 1/b-treated cells was significantly less than that of the non-targeting control siRNA-treated cells (* $p<0.05$) based on a one-way ANOVA with Dunnett t as the post hoc test. The experiment was repeated five times; however, DHS 0 siRNA treatment was included in the experiments only three times.

3.5 Cell proliferation during invasion

One possible explanation for the differences in invasion following treatment with various siRNAs is that cells treated with DHS siRNAs may proliferate differently from the control siRNA-treated cells. Such differences could register as differences in invasiveness in the Matrigel transwell experiments if cells were placed on the top of the inserts in serum-containing medium. Accordingly, in order control for this possibility, in these experiments, cells were placed in completely serum-free medium.

To confirm the effect of the serum-free medium on cell proliferation, cells were seeded on 96-well plates at the same time as corresponding cells were placed on the top of the Matrigel membrane in the invasion inserts, and XTT assays were performed at 12, 36, 43, and 57 hours after the counterpart cells were placed on Matrigel (Figure 10). In order to provide a reference for the absorbance values in Figure 10, and to elucidate the relationship between absorbance values and cell density in PC3 cells, a standard curve with absorbance and PC3 cell densities was created, as illustrated in Figure 11. The standard curve shows that the relationship between cell

Figure 10. Cell proliferation time course in the absence of serum during the period of the invasion assay.

PC3 cells were treated with DHS 0 siRNAs, DHS 1/b siRNAs, non-targeting control2 siRNAs or with GC7 for 48 hours. Then, the treated cells were seeded onto 96-well plates in triplicate at the same time corresponding cells were placed on the top of the Matrigel in invasion transwells, and cell proliferation was measured using XTT at 12 hours, 36 hours, 43 hours, and 57 hours after seeding. The results (absorbance values of means at 490 nm adjusted for background absorbance measured at 630 nm) are expressed as means +/- standard error. The standard curve of XTT cell proliferation shows a linear relationship between absorbance values and cell numbers of PC3 cells (see Figure 11). The results were analyzed with two-way ANOVA followed by Tamhane as the post hoc test. The analysis shows no significance among the treatment conditions during the period of the invasion assays.

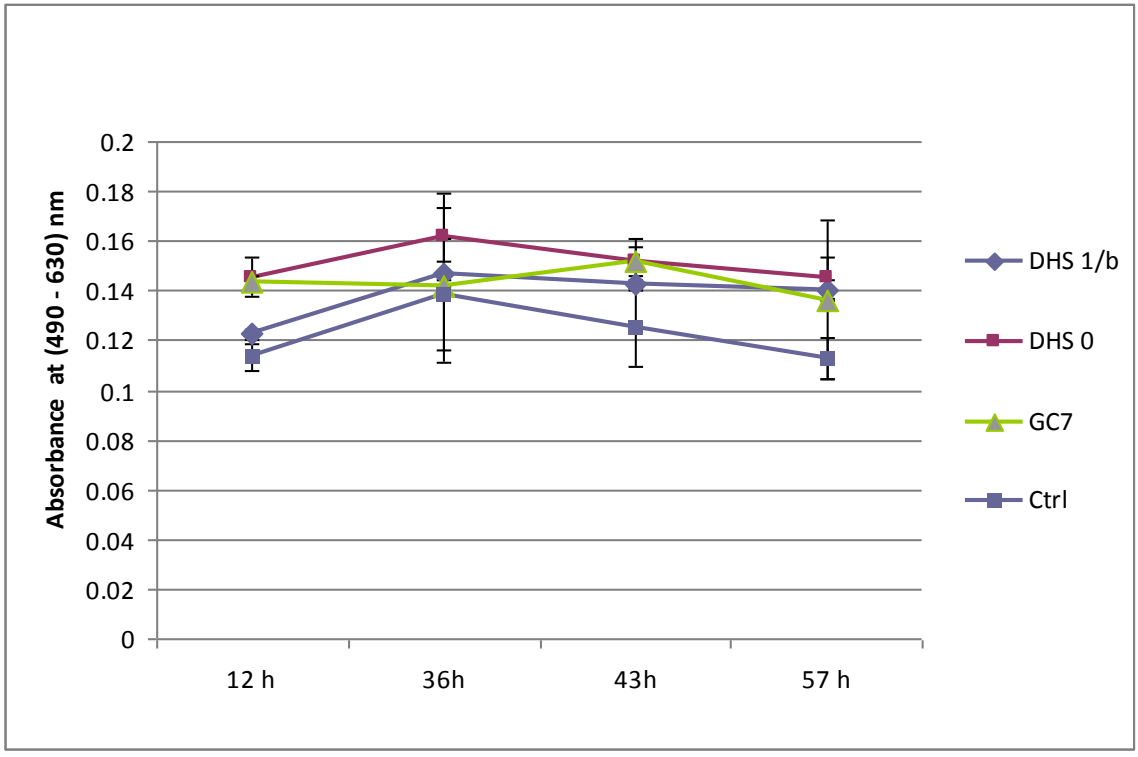
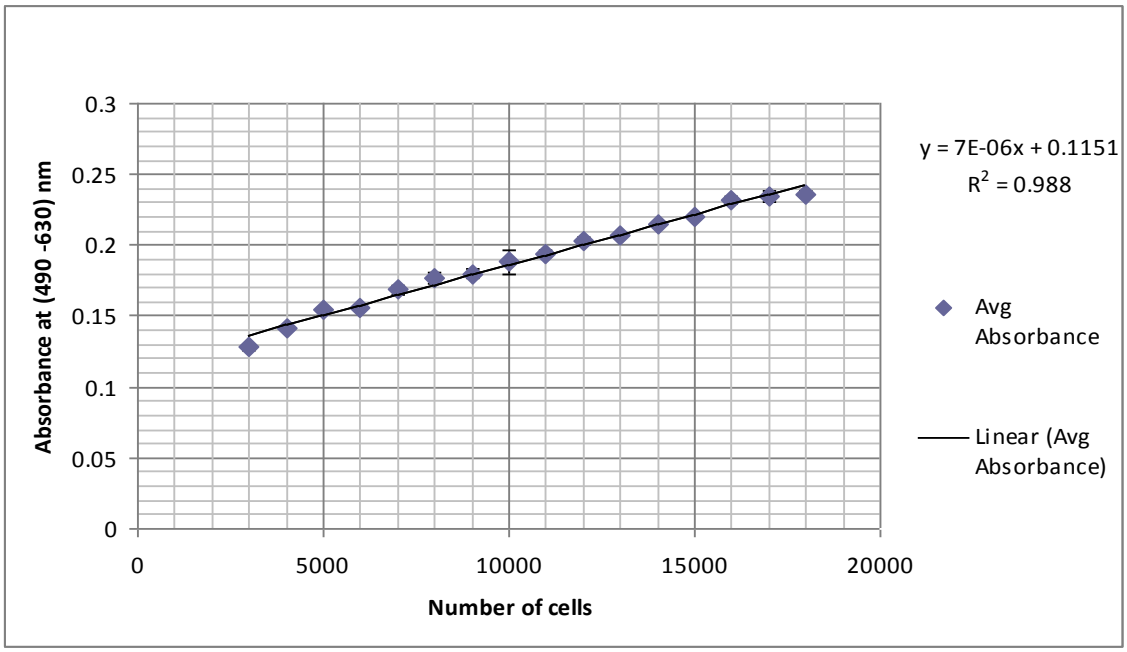

Absorbance at (490 - 630) nm
0.2
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
12 h
36h
43h
57 h
DHS 1/b
DHS 0
GC7
Ctrl

Figure 11. Standard curve for the XTT assay of cell proliferation using PC3 cells.

A standard curve was created to assess the relationship between absorbance values and cell number for the XTT cell proliferation assay. PC3 cells were seeded onto 96-well plates in increasing cell numbers, and XTT reagent mix was added to the wells 12 hours after seeding. The absorbance values were measured at 490 nm, with a background correction measured at 630 nm, three hours after the addition of the XTT reagent using a microplate reader. The linear standard curve of y=7*10E-6x + 0.1151 fit the experimental data with an r^2 value of 0.988.


0.3
0.25
0.2
0.15
0.1
0.05
0
Absorbance at (490 -630) nm
0
5000
10000
15000
20000
Number of cells
y = 7E-06x + 0.1151
R2 = 0.988
Avg Absorbance
Linear (Avg Absorbance)

number and absorbance values is linear. Specifically, the linear standard curve of y=7*10E-6x + 0.1151 fits the experimental data with an r^2 value of 0.988.

Cell proliferation results of the various treatment conditions are presented in Figure 10. A two-way ANOVA with Tamhane as the post hoc test showed no significant differences among the various treatment conditions. The results indicate that serum starvation effectively prevented cell proliferation during the period of the invasion assay.

3.6 Cell proliferation in the presence of serum

Cell proliferation under normal, 10% FBS growing conditions was measured using both cell counting using Trypan blue and the XTT cell viability/proliferation assay. For cell counting, PC3 cells were treated with DHS 0 siRNAs, DHS 1/b siRNAs, non-targeting control siRNAs, Lipofectamine 2000 alone (Mock), and GC7 for 96 hours, and cells were counted at 24 hours, 72 hours, and 96 hours after transfection (Figure 12). Cells were trypsinized, diluted with full medium, diluted with Trypan blue 1:2, and counted with a hemocytometer under a microscope. The experiment was done in duplicate. The clear, viable, cells were counted. The results were analyzed using two-way ANOVA with Fisher's LSD as the post hoc test. Statistical analysis of the results shows that both the DHS 0 siRNA-treated cells and the DHS 1/b siRNA-treated cells proliferated significantly less ($p<0.05$) than the control siRNA-treated cells. Moreover, the DHS 0 siRNA-treated cells, the DHS 1/b siRNA-treated cells, and the GC7-treated cells all proliferated significantly less than the untreated cells ($p<0.05$).

For the XTT cell proliferation assays, PC3 cells were treated with DHS 0 siRNAs, DHS 1/b siRNAs, non-targeting control siRNAs, Lipofectamine 2000 alone (Mock), or GC7 for 72

Figure 12. Cell proliferation time course measurement in the presence of serum using Trypan blue. PC3 cells were treated with DHS 0 siRNAs, DHS 1/b siRNAs, non-targeting control2 siRNAs, Lipofectamine 2000 alone (Mock), or GC7 for 96 hours, and cell proliferation was measured at 24 hours, 72 hours, and 96 hours after transfection. Cells were trypsinized, and the cell suspensions were diluted with medium supplemented with 10% FBS, diluted with Trypan blue 1:2, and the cells were counted with a hemocytometer under a microscope. The experiment was done in duplicate. The viable cells were counted. The results are expressed as average percentages of untreated cells +/- standard error. The results were analyzed using a two-way ANOVA with Fisher's LSD as the post hoc test. DHS 0 siRNA-treated cells and DHS 1/b siRNA treated cells were found to proliferate significantly less ($p<0.05$) than control siRNA-treated cells. DHS 0 siRNA-treated cells, DHS 1/b siRNA-treated cells, and GC7-treated cells proliferated significantly less than the untreated cells ($p<0.05$).

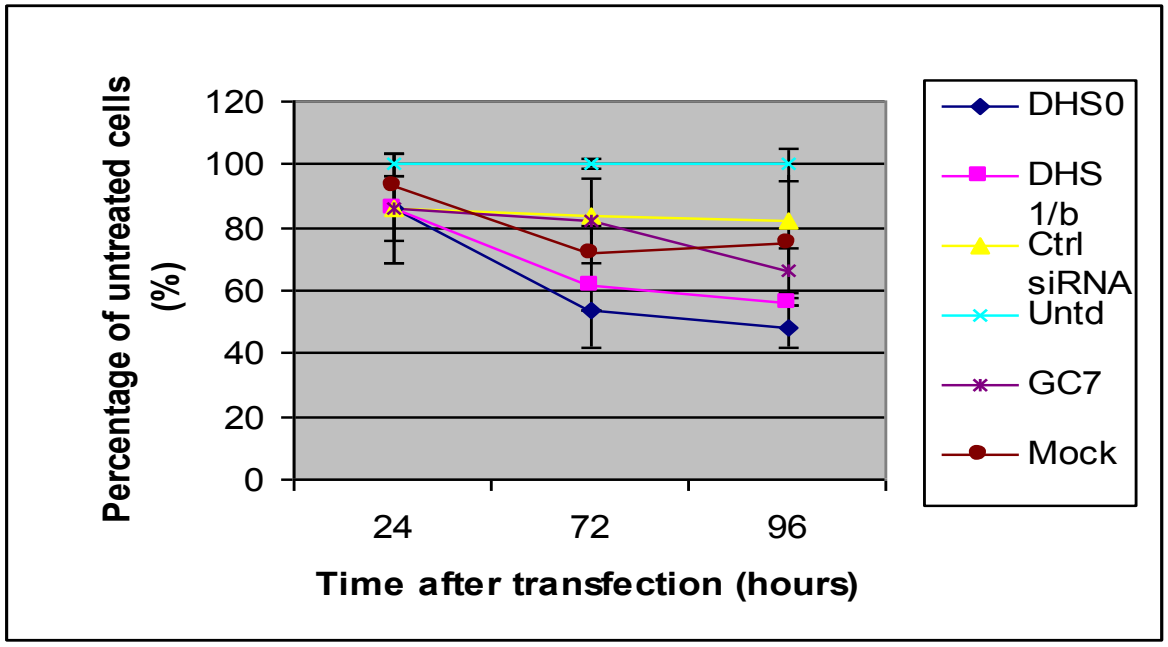
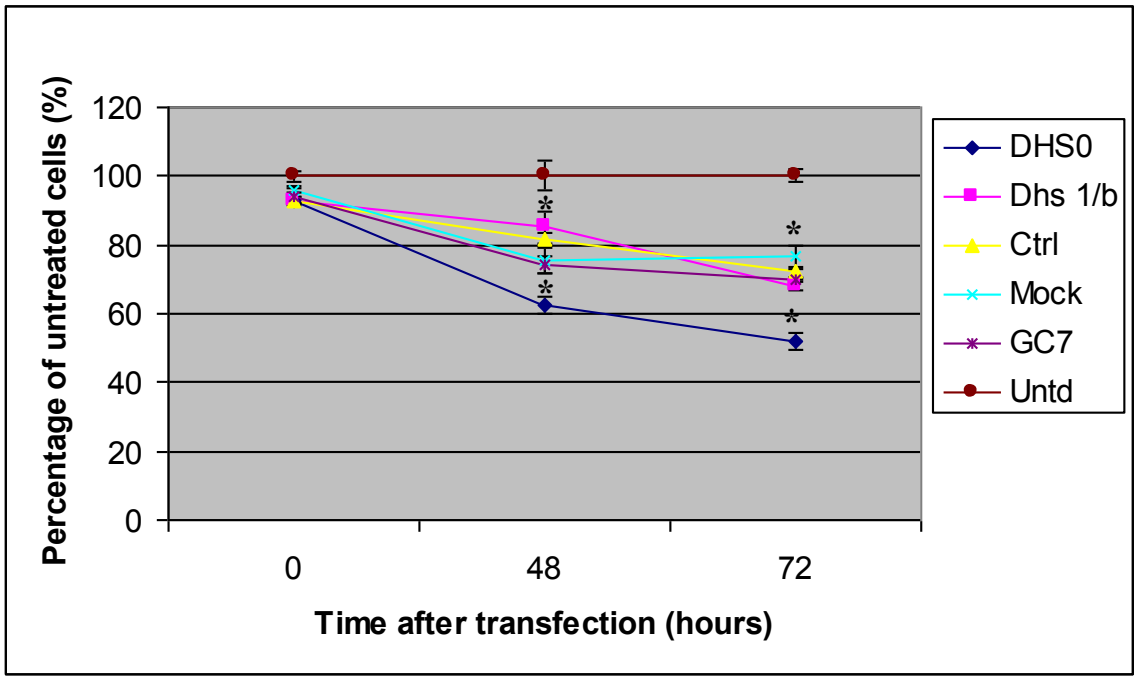

Percentage of untreated cells (%)
120
100
80
60
40
20
0
24
72
96
Time after transfection (hours)
DHS0
DHS
1/b Ctrl siRNA
Untd
GC7
Mock

Figure 13. Cell proliferation time course measurement in the presence of serum using XTT.

PC3 cells were treated with DHS 0 siRNAs, DHS 1/b siRNAs, non-targeting control2 siRNAs, Lipofectamine 2000 alone (Mock), or GC7 for 72 hours, and cell proliferation was measured at 0h, 48 hours, and 72 hours after transfection. The experiment was done in triplicate. The results are expressed as average percentage of untreated cells +/- standard error. The results were analyzed with a two-way ANOVA and Fisher's LSD and as the post hoc test. Fisher's LSD showed that all treatment conditions were significantly different from the untreated cells ($p<0.05$). DHS 0-siRNA treated cells also proliferated significantly less than the control siRNA-treated cells ($p<0.01$) at both 48 and 72 hours.


Percentage of untreated cells (%)
120
100
80
60
40
20
0
0
48
72
Time after transfection (hours)
DHS0
Dhs 1/b
Ctrl
Mock
GC7
Untd

hours, and cell proliferation was measured at 0 hour, 48 hours, and 72 hours after transfection (Figure 13). The experiment was done in triplicate. The results were analyzed with two-way ANOVA and Fisher's LSD as the post hoc test. All treatment conditions proliferated significantly less than the untreated cells ($p<0.05$). Furthermore, DHS 0 siRNA-treated cells proliferated significantly less than the control siRNA-treated cells ($p<0.01$).

A separate experiment, when cell proliferation was measured only at 72 hours after transfection using the XTT assay, was also conducted (Figure 14). PC3 cells were treated with DHS 0 siRNAs, DHS1/b siRNAs, non-targeting control1 siRNAs, or GC7 for 72 hours. The experiment was done using six wells for each condition. Statistical analysis was done using one-way ANOVA with Fisher's LSD as the post hoc test. All of the siRNA treated samples, including the control1 siRNA, together with the GC7 treatment show significant suppression of cell proliferation relative to the untreated cells. However, since the level of suppression in the presence of the DHS 0 siRNAs was not significantly different from the control1 siRNA treatment, and the DHS 1/b siRNA-treated cells proliferated significantly more than the control1 siRNA-treated cells, the results from this experiment are inconclusive.

3.7 Apoptosis measurement

To assess the ability of the DHS siRNAs to induce apoptosis in PC3 cells, the Annexin V/PI apoptosis assay and flow cytometry were used. PC3 cells were treated with DHS 0 siRNAs, DHS 1/b siRNAs, non-targeting control2 siRNAs, Lipofectamine 2000 alone (Mock), or GC7 for 96 hours, or placed in serum-free medium for 72 hours, and apoptosis was measured at 96 hours

Figure 14. Cell proliferation assay in the presence of serum using XTT.

PC3 cells were treated with DHS 0 siRNAs, DHS1//b siRNAs, non-targeting control1 siRNAs, or GC7 for 72 hours, and cell proliferation was measured at 72 hours after transfection using the XTT assay. The experiment was done using six wells for each condition. The results are expressed as average percentage of untreated cells +/- standard error. Statistical analysis using a one-way ANOVA with Dunnett t as the post hoc test indicated that all treatment conditions showed significantly lower levels of cell proliferation than the untreated cells ($p<0.05$). Fisher's LDS showed that both DHS 0 siRNA-treated cells and DHS 1/b siRNA-treated cells proliferated significantly less ($p<0.01$) than the positive control, GC7-treated cells, which in turn, proliferated significantly less than the untreated cells (* $p< 0.01$). However, DHS 1/b-treated and GC7-treated cells proliferated significantly more than the control siRNA-treated cells.

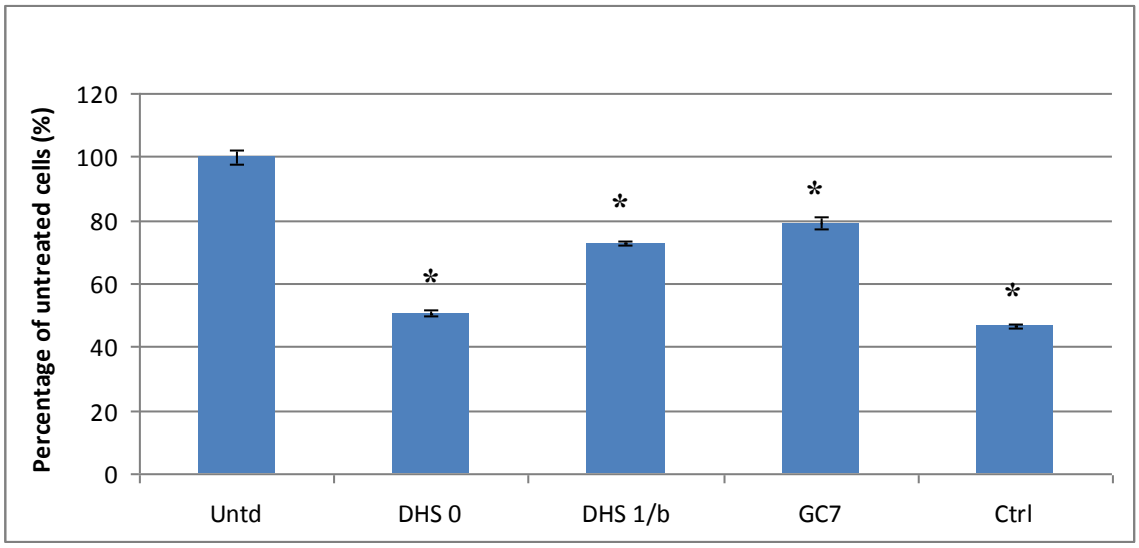
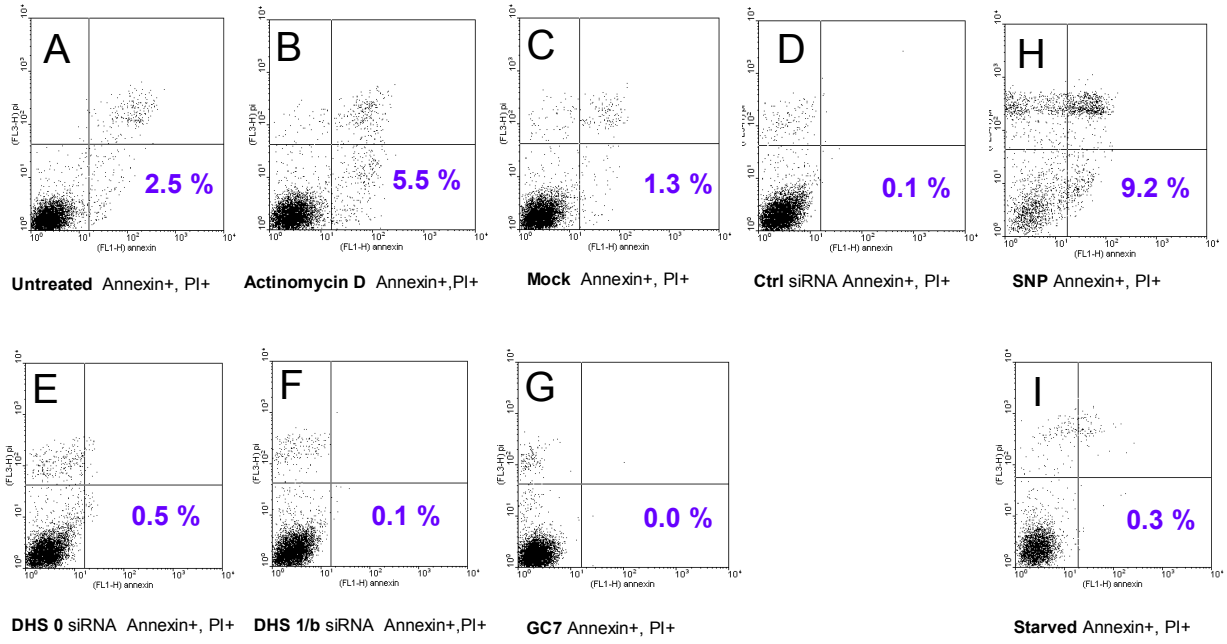

Percentage of untreated cells (%)
120
100
80
60
40
20
0
*
*
*
*
Untd
DHS 0
DHS 1/b
GC7
Ctrl

Figure 15. Apoptosis measurement in PC3 cells using the Annexin V/PI apoptosis assay.

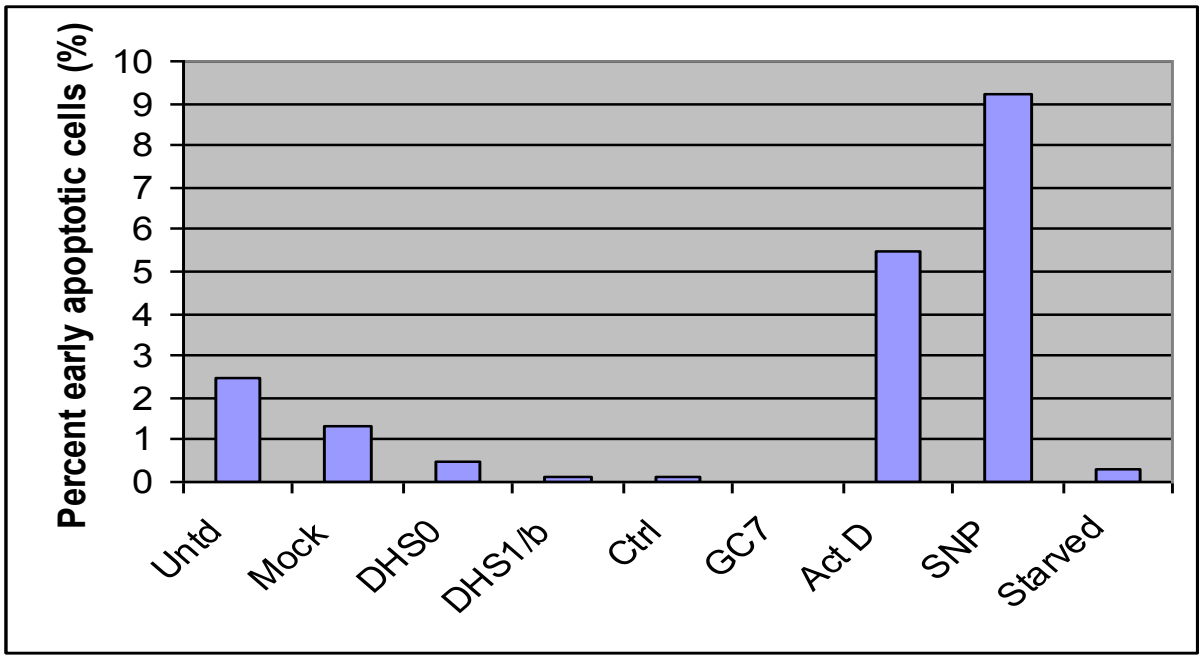
PC3 cells were treated with DHS 0 siRNAs, DHS 1/b siRNAs, non-targeting control siRNAs, Lipofectamine 2000 alone (Mock), or GC7 for 96 hours, or placed in serum-free medium for 72 hours. Apoptosis was measured 96 hours after treatment. As a positive control, PC3 cells were treated with 0.5 μ/ml Actinomycin D or 10mM sodium nitroprusside (SNP). The SNP and starvation (Panel A, Dot blots H and I) treatment conditions are from a separate experiment. The results are expressed as percent of early apoptotic cells. Panel A. Dot blots of the various conditions. The lower left quadrant shows viable cells, the lower right quadrant shows early apoptotic cells, the upper right quadrant shows late apoptotic and dead cells (including secondary necrotic cells). In the upper left quadrant, cells with damaged cell membrane (Annexin V-negative and PI-positive) can be seen. Panel B. The bar graph shows the percentage of early apoptotic cells.

Panel A.



Untreated Annexin+, PI+ **Actinomycin D** Annexin+,PI+ **Mock** Annexin+, PI+ **Ctrl** siRNA Annexin+, PI+ **SNP** Annexin+, PI+

DHS 0 siRNA Annexin+, PI+ **DHS 1/b** siRNA Annexin+,PI+ **GC7** Annexin+, PI+ **Starved** Annexin+, PI+



after transfection (Figure 15). As positive controls, PC3 cells were treated with 0.5 μg/ml Actinomycin D for 48 hours or 10mM sodium nitroprusside (SNP) for 24 hours.

The dot blots in Figure 15 show living cells and cells in the various dying stages. The lower left quadrants show viable cells, the lower right quadrants show early apoptotic cells, and the upper right quadrants show late apoptotic and already dead cells (including secondary necrotic cells). In the upper left quadrant, cells with damaged cell membrane (Annexin V-negative and PI-positive) can be seen.

The results indicate that both DHS 0 siRNA-treated and DHS 1/b siRNA-treated cells showed less than 1% early apoptosis (Figure 15). Similarly, control2 siRNA-treated cells and GC7-treated cells also had less than 1% early apoptotic cells. The positive control, Actinomycin D induced 5.5% apoptosis, while the SNP-treatment induced 9.2 % apoptosis in PC3 cells. The untreated cells showed 2.5% apoptosis, while the Lipofectamine 2000 treatment alone (Mock) induced 1.3% apoptosis. The experiment was repeated twice with similar results. In sum, the results of the experiments indicate that DHS siRNA-treatment does not induce apoptosis in PC3 cells under the tested experimental conditions.

Chapter 4
Discussion

The objectives of this study were to investigate the role of deoxyhypusine synthase (DHS) in cell invasiveness, cell proliferation, and apoptosis in cancer cells using RNA interference. DHS is the first enzyme in the hypusination pathway of eIF5A, and it catalyzes the transfer of the 4-aminobutyl moiety of spermidine to $Lysine_{50}$ of the lysine form of eIF5A, L-eIF5A, forming a deoxy-hypusinated form of eIF5A (D-eIF5A) (Park, 2006). The hypusination reaction of eIF5A is completed by deoxyhypusine hydroxylase, DHH, which places a hydroxyl group on C-2 of the 4-aminobutyl moiety of D-eIF5A, resulting in the final hypusinated form of eIF5A (H-eIF5A) (Park, 2006).

eIF5A has two isoforms in humans, eIF5A-1 and eIF5A-2, which are 84 percent identical at the protein level (Jenkins et al., 2001). DHS catalyzes the hypusination of both eIF5A-1 and eIF5A-2. Whereas eIF5A-1 is ubiquitously expressed in mammalian cells, eIF5A-2 is much less frequently expressed at high levels. For example at the protein level, eIF5A-2 is only highly expressed in certain cancer cells, such as certain ovarian and colon cancer cells, and it has been proposed to function as an oncogene (Guan et al., 2004). Hypusinated eIF5A-2 is the most likely form of eIF5A-2 to function as an oncogene since eIF5A-2 was found in the hypusinated form in cancer cells that highly express eIF5A-2 at the protein level, such as UACC-1598 cells (Clement et al., 2003). It has been suggested that the two eIF5A isoforms may have redundant functions (Eshaque, 2006). PC3 cells, the primary experimental system of the present study, express eIF5A-2 protein at a very low level (Clement et al., 2005).

In addition to its catalytic role in the hypusination of eIF5A, DHS has also been proposed to play a regulatory role in eIF5A hypusination (Kang and Chung, 2003; Kung et al., 2002; Thompson et al., 2003). DHS has been found to be phosphorylated by protein kinase C and protein kinase CK2 (Kang and Chung, 2003). Based on its phosphorylation and its strategic role in the hypusination of eIF5A, DHS was suggested to regulate the relative abundance of the various forms of eIF5A. Thompson et al. (2003) found evidence for the proposed regulatory role of DHS when they showed that the N-terminal α-helix can regulate DHS's interaction with L-eIF5A by modulating L-eIF5A's access to the active site of DHS. It is proposed that DHS regulates cell survival and cell death through its role in regulating the relative amount of L-eIF5A and H-eIF5A in cells, since the ratio of H-eIF5A to L-eIF5A is proposed to be the determining factor in whether cells live or die (Thompson, personal communication). The rate of accumulation of each form, H-eIF5A and L-eIF5A, is a function of the rates at which eIF5A is synthesized, hypusinated and degraded (Thompson, personal communication). Thus, DHS may play a key role in regulating cell survival and cell death through the hypusination pathway of eIF5A.

Deoxyhypusine synthase has also been shown to be involved in metastasis. It was found to be one of eight signature metastatic genes in human cancers (Ramaswamy et al., 2003). Since DHS catalyzes the hypusination of eIF5A, DHS is likely to exert its metastatic effect through its catalytic role in the hypusination pathway of eIF5A. Furthermore, DHS's role in metastasis is further supported by evidence that the hypusinated form of eIF5A-2 has been linked to metastasis in colon, ovarian, and gastric cancers (Guan et al., 2004; Marchet et al., 2006; Xie et al., 2008), and the findings of the present study also confirm this role.

The role of H-eIF5A-1 in survival is well established (Park, 2006; Park et al., 1998; Schnier et al., 1991). A number of studies have shown that H-eIF5A-1 plays a role in cell proliferation in yeast and mammalian cells since the inhibition of the hypusination of L-eIF5A has resulted in decreased cell division (Chen et al., 1996; Clement et al., 2002; Lee et al., 2002; Park et al., 1994; Tome and Gerner, 1997). However, Taylor et al. (2007) showed that eIF5A-1 depletion by >90% using RNAi (the depletion of the H-eIF5A-1) resulted in no effect on cell proliferation or cell viability. Nevertheless the residual level of H-eIF5A-1 in these eIF5A-1 siRNA-treated cells may still have been enough to support cell proliferation (Taylor et al., 2007). By contrast, H-eIF5A-2 has been associated with cell proliferation (Thompson et al., 2004; Clement et al., 2003). Furthermore, the unmodified form of eIF5A-1 has been shown to induce apoptosis (Taylor et al., 2007).

The main finding of the present study is that both DHS 1/b siRNA treatment and DHS 0 siRNA treatment significantly decreased cell invasiveness in PC3 cells, a highly metastatic prostate cancer cells, compared to the non-targeting control siRNA-treated cells ($p<0.05$). The similar results obtained by the two DHS siRNAs that target different parts of the DHS mRNA strengthen the conclusion that a decrease in DHS results in decreased invasiveness in metastatic cancer cells. Potential alternative explanations and confounding factors were controlled for, or measured, during the invasiveness experiments. Placing cells in serum-free medium successfully controlled for potential differences in cell proliferation among the various treatment conditions. Thus, cell proliferation differences are not likely to account for the differences in invasiveness. Apoptosis was also measured to evaluate whether it contributed to the differences in cell invasion among the various treatments. Since DHS siRNA treatment did not induce apoptosis in

PC3 cells, apoptosis is also very unlikely to contribute to the invasiveness results. Consequently, DHS siRNA-treatment appears to effectively reduce invasiveness in PC3 cells compared to the non-targeting control siRNA-treated cells. These results confirm the hypothesis that reduction of DHS expression results in decreased invasiveness in metastatic cancer cells.

The results from the invasiveness experiments in the present study are consistent with the role of DHS as a metastatic marker, as shown by Ramaswamy et al. (2003), since inhibition of DHS inhibited the invasiveness of metastatic cancer cells. Since inhibition of DHS results in the simultaneous accumulation of the unmodified form of eIF5A and a decrease in the hypusinated form of eIF5A, it is likely that the decreased invasiveness of highly metastatic cancer cells as a result of DHS inhibition was due to the decrease in the hypusinated form of eIF5A. Thus, the hypusinated form of eIF5A is likely to play a role in metastasis, and the metastatic role of DHS is likely to be exerted through its regulatory role in the hypusination pathway of eIF5A.

Since PC3 cells express the eIF5A-2 protein at a very low level, it is more likely that the hypusinated form of eIF5A-1 is involved in metastasis. Furthermore, the findings that eIF5A2 is only expressed at high levels in certain colon and cervical cancers, and that in the majority of highly metastatic cancers eIF5A-2 expression does not correlate with metastasis, suggest that H-eIF5A-2 may play a significant role in metastasis in only those cancers in which it is over-expressed (Clement et al., 2006). H-eIF5A-1 and 2 may also be functionally redundant and substitute for one another (Eshaque, 2006). Alternatively, although unlikely, the reduction in the small amount of H-eIF5A-2 in PC3 cells could be responsible for the decreased invasiveness in response to DHS inhibition in the current study. Further experiments in which eIF5A-2 is

completely depleted using siRNA against eIF5A-2 mRNA could confirm that the decreased invasiveness in PC3 cells is due to changes in the various firms of eIF5A-1 and not eIF5A-2.

As mentioned earlier, it was important to ensure in the cell invasion experiments that the decrease in cell invasiveness upon DHS suppression was not attributable to an effect of DHS siRNA treatment on cell proliferation. To control for this, the cell invasiveness assay was carried out in serum-free medium. XTT assays measuring cell proliferation confirmed that there was no significant cell proliferation in the absence of serum. Under serum-free conditions, DHS siRNA treatments did not result in significant decrease in cell proliferation in PC3 cells during the period of the invasion experiments. Thus, these results confirmed that cell proliferation differences were not likely to confound the invasiveness results.

In addition, the effect of DHS suppression on cell proliferation was examined in the presence of serum. The effects of DHS treatment on cell proliferation under normal serum conditions (10% serum supplementation) suggest that inhibiting DHS may result in decreased cell proliferation. The results from a 96-hour cell proliferation time course conducted using Trypan blue indicate that DHS siRNA-treated cells, both DHS 0 siRNA and DHS 1/b siRNA, resulted in significantly less cell proliferation than the non-targeting control siRNA-treatment ($p<0.05$). Similarly, measurement of cell proliferation at various time points over 72 hours using XTT indicated that DHS 0 siRNA-treatment resulted in less cell proliferation compared to non-targeting control siRNA-treatment ($p<0.05$), although the DHS 1/b siRNA-treatment did not result in significantly different cell proliferation from the control siRNA treatment. Results from another XTT cell proliferation measurement at 72 hours after transfection were inconclusive since the non-targeting control siRNA treatment resulted in significantly less cell proliferation

than the DHS 1/b siRNA treatment and the positive control GC7 treatment. As these experiments on cell proliferation in the presence of serum were performed only once, mostly in triplicate or using six wells, the evidence for DHS siRNA treatment decreasing cell proliferation would be strengthened by repeating the experiments.

Nevertheless, taken together, the evidence from the cell proliferation experiments in the current study seems to suggest that DHS siRNA treatment may decrease cell proliferation under normal serum conditions in PC3 cells. Thus some of the experimental findings confirm the research hypothesis that inhibition of DHS inhibits cell proliferation, although others are inconclusive. This is, in fact, consistent with conflicting reports in the literature, some indicating that DHS suppression inhibits cell proliferation, others indicating that DHS suppression or the depletion in H-eIF5A has no effect on cell proliferation (Chen et al., 1996; Shi et al., 1996; Park, 2006; Taylor et al., 2007). These observations also suggest that the effect of DHS suppression on cell proliferation may vary from cell line to cell line. It would appear from the results of the present study that if there is an effect of DHS suppression on cell proliferation, it is likely to be relatively small. It is also possible that the inhibition of DHS results in the accumulation of spermidine, and thus the polyamine pathway is affected and possibly compensates for the decrease in the H-eIF5A and supports cell proliferation. In sum, the cell proliferation observations suggest that there may be an effect of DHS suppression on cell proliferation but no significant conclusions can be drawn from the data obtained.

It was also important to ensure that the decrease in cell invasion observed upon suppression of DHS in the invasiveness experiments was not attributable to the induction of apoptosis. Thus using the Annexin V/PI apoptosis assay, apoptosis was measured in PC3 cells

treated with two DHS siRNAs, DHS 0 siRNAs and DHS 1/b siRNAs. The results indicate that PC3 cells did not indicate significant apoptosis in the various treatment conditions. DHS 0 siRNA treatment, DHS 1/b siRNA treatment, and the non-targeting control siRNA treatment all induced less than one percent apoptosis in PC3 cells. The Actinomycin D-treatment, which served as a positive control, induced 5.5% early apoptosis, suggesting that the assay was performed properly. In a separate experiment, an alternate positive control, sodium nitroprusside induced 9% early apoptosis in PC3 cells also confirming that the assay worked properly, and the other treatment conditions showed similar results as described above.

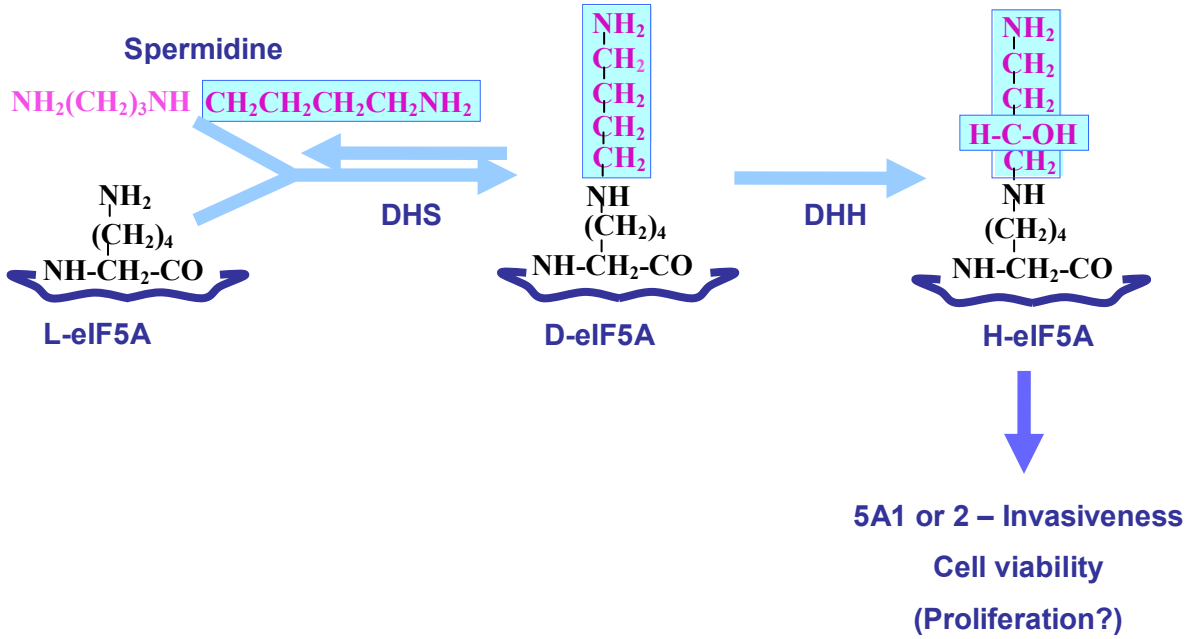
Thus, these results suggest that the various siRNA treatments were unable to induce apoptosis in PC3 cells, which is not consistent with previous research findings which suggest that inhibition of DHS results in apoptosis (Huang et al., 2007). The different results may be due to differences in cell lines, or possibly, a small amount of H-eIF5A may be enough to support cell survival, and with a longer treatment and more efficient depletion of the H-eIF5A, DHS siRNA treatment may induce apoptosis. Nevertheless, in the present study, the lack of apoptosis induced by DHS siRNA-treatment served as a control and suggested that the results of the invasiveness experiments cannot be attributed partly to apoptosis induced in the DHS siRNA-treated cells. On the other hand, the results of the present study are consistent with the reports of some research articles that reported that suppression of DHS with GC7 results in inhibited cell proliferation, resulting in cytostasis, but they did not report apoptosis (Lee et al., 2002). Furthermore, the differing results could be explained by the ratio at which the L-eIF5A accumulates in cells relative to H-eIF5A, which induces apoptosis when the level of L-eIF5A exceeds the level of H-eIF5A. The relative amount of the H-eIF5A and the L-eIF5A, in turn,

depends on the rate of eIF5A synthesis and degradation, which is likely to be different in different cell lines. Consequently, the results from this study suggest that in PC3 cells DHS siRNA treatment does not induce apoptosis.

Taken together, the results from the present study suggest the following model for DHS and eIF5A functions (Figure 16). Since the reduction of DHS levels resulted in decreased invasiveness of PC3 cells and a concurrent decrease in the hypusinated eIF5A, the hypusinated eIF5A is likely to be involved in metastasis. It may do so by playing a role in the translation or shuttling of a subset of mRNAs that are involved in cancer cell invasiveness, such as cadherins and other cell adhesion molecules. For instance, N-cadherin has been found to be up-regulated during tumor progression and to induce invasiveness, both *in vitro* and *in vivo,* through its interaction with fibroblast growth factor 2 (FGF-2) and the resultant activation of the MAPK-ERK pathway. This activation, in turn, leads to up-modulation of matrix metalloproteinase 9 (MMP9) and increased invasiveness (Hazan et al., 2000; Hazan et al., 2004; Suyama et al., 2002). However, depletion of all post-translationally modified forms of eIF5A-1 with RNAi did not result in significant changes in invasiveness (pilot study, data not shown). Since DHS hypusinates both eIF5A-1 and eIF5A-2, and although PC3 cells express eIF5A-1 at a high level and a very low level of eIF5A-2 is also present in this cell line, there could have been sufficient hypusinated eIF5A-2 present after eIF5A-1 depletion to influence invasiveness. Thus, the hypusinated form of either eIF5A-1 or 2 could play an important role in cancer cell invasiveness.

Figure 16. Proposed functions of DHS and the various forms of eIF5A.

Based on the results from the present study, H-eIF5A-1 is likely to be associated with cancer cell invasiveness since a decrease in DHS levels resulted in a decrease in the hypusinated form of eIF5A-1 and a concurrent decrease in the invasiveness of metastatic cancer cells.


Spermidine
NH2(CH2)3NH
CH2CH2CH2CH2NH2
DHS
DHH
NH2
(CH2)4
NH-CH2-CO
L-eIF5A
NH
(CH2)4
NH-CH2-CO
D-eIF5A
CH2
H-C-OH
H-eIF5A
5A1 or 2 – Invasiveness
Cell viability
(Proliferation?)

As the reduction of DHS levels indicated a reduction in cell proliferation, in some experiments although not in others, it is possible that either the H-eIF5A-1 is involved in cell proliferation, or alternatively, the decrease in the already very small amount of H-eIF5A-2 might have caused the observed reduction in the cell proliferation. However, the latter possibility is unlikely because of the very small amount of eIF5A-2 protein in PC3 cells. The proposed role of H-eIF5A-1 in cell proliferation is in line with previous research results (Park et al., 1994; Park, 2006; Lee et al., 1998; Clement et al., 2002), although Taylor et al. (2007) found that H-eIF5A was not essential for cell proliferation.

In summary, the results from the present study indicate that the DHS siRNA treatment reduces invasiveness of PC3 cells, probably through the inhibition of the hypusination of eIF5A. Furthermore, DHS siRNA treatment appears to decrease cell proliferation in the presence of serum; however, since some cell proliferation experimental results in the present study were inconclusive, further research into the effect of DHS inhibition on cell proliferation is necessary to draw more definitive conclusions. In addition, DHS siRNA treatment does not induce apoptosis in PC3 cells under the tested conditions, although previous research findings from the Thompson Laboratory indicate that the over-expression of L-eIF5A induces significant apoptosis in cancer cells (Taylor et al., 2007). The effects of DHS siRNA-treatment on metastatic cancer cell invasiveness, and perhaps on cell proliferation, provide supporting evidence for the possibility of using DHS depletion in potential cancer treatments. Depletion of DHS in conjunction with the over-expression of L-eIF5A is likely to yield the best results since DHS depletion would reduce invasiveness and perhaps reduce cell proliferation while over-expression of L-eIF5A resulting in the relative overabundance of L-eIF5A compared to H-eIF5A would induce apoptosis in cancer cells.

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