FUNCTION OF HEMATOPOIETIC- AND NEUROLOGIC-EXPRESSED SEQUENCE 1 (HN1) IN CANCER

By

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I dedicate this work to my dad, mom, brother and four grandparents.
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The hematopoietic- and neurologic-expressed sequence 1 (Hn1) gene encodes a small protein that is highly conserved among species. Its expression is upregulated in regenerating neural tissues, including the axotomized adult rodent facial motor nerve and dedifferentiating retinal pigment epithelial cells of the Japanese newt. Hn1 is also expressed in multiple developing murine tissues and regions of the adult rat brain that exhibit high plasticity, and it has been reported as a marker for human ovarian carcinoma. The goal of this study was directed toward understanding the function of Hn1 in cancer cells. Hn1 expression was identified in murine GL261 glioma and B16.F10 melanoma cells and tumors. It was also detected in numerous human cancer cell lines including esophageal and ovarian carcinomas, and erythroleukemia, as well as in human high-grade gliomas. An adeno-associated virus (AAV) expressing anti-Hn1 siRNA was developed to study the effects of depleting Hn1 protein in cancer cells. Hn1 depletion in GL261 cells caused reduced tumor volumes. In the B16.F10 cells, it induced cell cycle arrest and increased melanogenesis and secretion. Changes were also evident in the expression and phosphorylation levels of proteins involved in melanogenesis and cell cycle progression, including increased p38 phosphorylation (P-p38) and reduced ERK.
phosphorylation (P-ERK). The microphthalmia transcription factor (MITF), which is known to drive melanocytic differentiation by increasing the expression of melanogenic enzymes and arresting the cell cycle, is upregulated by P-p38 and downregulated by P-ERK. However, contrary to what was expected, MITF was reduced in Hn1-depleted cells, as was the related transcription factor USF-1. Treatment of Hn1-depleted cells with cAMP-elevating agents prevented MITF downregulation, as did inhibition of p38 and MEK signaling. The expression of TFE3, another transcription factor that had been reported in the regulation of melanogenesis and in cancer, was elevated in the absence of Hn1. This suggested that Hn1 protein in B16.F10 cells may normally inhibit the function of TFE3 to prevent differentiation. Collectively, these data, coupled with reports on Hn1 expression in regeneration and development, indicate that Hn1 is involved in the regulation of cell differentiation. This hypothesis was further tested in RAW264.7 and HEK293 cells.
CHAPTER 1
INTRODUCTION

Hematopoietic- and Neurologic-Expressed Sequence 1

The hematopoietic- and neurologic-expressed sequence 1 (Hn1) gene was first reported twelve years ago (Tang et al. 1997). DNA sequences encoding Hn1 were isolated from a cDNA library corresponding to primitive erythroid cells derived from murine yolk sac blood islands on the ninth day of gestation. These investigators observed that hematopoietic cells and fetal brain expressed the highest levels of Hn1 mRNA, and they gave the gene its name based on the postulate that it is important in neurological and hematopoietic development. Hn1 has a short and unique protein sequence consisting of 154 amino acids (Tang et al. 1997) that is highly conserved among humans, rodents, primates, cattle, birds, fish, amphibians, and insects (NCBI) (Figure 1-1). Murine Hn1 is found on chromosome 11, which is often considered equivalent to human chromosome 17, where human HN1 is located (locus 17q25.2) (Tang et al. 1997). Chromosomal loss in locus 17q25 has been associated with sporadic breast tumors (Petty et al. 1996). Upstream and adjacent to HN1 are SUMO2 (small ubiquitin-like modifier) and NUP85 (nucleoporin85); downstream are NT5C (cytosolic deoxyribonucleotidase), ARMC7 (armadillo repeat containing 7), and SLC16A5 (monocarboxylic acid transporter). SUMO2, HN1 and NT5C are encoded in the 3’ to 5’ direction (Figure 1-2). The same genes are found together in murine chromosome 11 (NCBI). The human HN1 gene consists of 5 exons (Zhou et al. 2004). HN1 is also found in the NCBI records under the name “androgen-regulated protein 2” (ARM2), but this protein has not been discussed in any available scientific reports (NCBI).

Does the Amino Acid Sequence of Hn1 Provide Clues about Its Function?

Because of the uniqueness of its amino acid sequence, it is difficult to predict the cellular function of Hn1 based on comparisons with other proteins. The only other protein that has very
high sequence identity with HN1 is called HN1-Like (HN1-L), and its function is also unknown (Zhou et al. 2004). HN1 shares motifs, “PPGG” and “MASNIF,” with a protein named Jupiter, the ortholog of HN1-L in *Drosophila melanogaster*, which was identified as a microtubule-binding protein (Karpova et al. 2006) (Figure 1-2). The sequence PPGG resembles the PGGG motif found in numerous microtubule-binding proteins. The amino acid sequence of Hn1 contains three consensus sites for N-linked glycosylation and one for SUMOylation (Figure 1-1). Relative to the average protein, the sequence of Hn1 is very rich in small turn-promoting amino acids, notably proline, serine and glycine (2.2, 1.9 and 1.7 times the GenBank average respectively), and it is low in hydrophobic amino acids (Figure 1-3). In general, the composition of Hn1 is similar to that described for intrinsically unstructured proteins (Dyson and Wright 2005). Therefore, Hn1 may be functionally related to GAP43, MARCKS and several other intrinsically unstructured proteins which regulate cytoskeletal organization.

An extensive analysis comparing the phosphorylation states of proteins during G1 phase and mitosis in HeLa cells identified HN1 as one of many proteins that are phosphorylated at much higher levels during mitosis. Six phosphorylation sites were identified in HN1 at T54, S70/S80, S71, S80, S87, and S88 (Figure 1-4). Phosphorylation at S71 (S*GGR) matched one of the motifs, [pS/pT]-[A/G]-X-[K/R], that were newly identified as consensus sites that are commonly phosphorylated during mitosis (Dephoure et al. 2008). The multisite phosphorylation of proteins increases their regulatory potential. The identification of multiple HN1 phosphorylation sites suggests that this protein may undergo changes in conformation, interactions, stability, and subcellular localization (Cohen 2000).

In search of clues about the function of Hn1 based on its structure, a combination of software programs were used to draw the model for a predicted murine Hn1 structure. A program
called HHMSTR/Rosetta (Bystroff and Shao 2002; Bystroff et al. 2000) accessible online (URL: http://www.bioinfo.rpi.edu/~bystrc/hmmstr/server.php) was used to predict the three-dimensional coordinates for each amino acid in the murine Hn1 sequence. The predicted coordinates were entered on the software PyMol by DeLano Scientific LLC to obtain a 3D image of the predicted structure. This model was then energy-minimized using the Powell method. The finalized model for a predicted structure of murine Hn1 resembles a horseshoe or ring and the protein contains a few small \( \alpha \)-helices and \( \beta \)-sheets (Figure 1-4). This predicted model of the structure of murine Hn1 is based solely on its amino acid sequence, therefore it can only provide an idea of one possibility and cannot be claimed as the structure of Hn1 unless it is confirmed by X-ray crystallography.

**Hn1 Interactions**

A systematic yeast-two-hybrid analysis of human proteins screened using a matrix approach and two rounds of automated interaction mating, identified two proteins that interact physically with Hn1, a Rho-type GTPase-activator protein (Rho-GAP) called RICH2, and the splicing factor proline- and glutamine-rich (SFPQ) (Stelzl et al. 2005). Like HN1, RICH2 is located in human chromosome 17, locus 17p12. Its mRNA is highly expressed in human brain, where expression of its related protein, RICH1, is very low. RICH2 binds the Rho GTPases CDC42 and RAC1 and activates their GTP hydrolysis (Richnau and Aspenstrom 2001). CDC42 and RAC1 have multiple functions. They can regulate the actin cytoskeleton, mitogen-activated protein kinase (MAPK) cascades, cell cycle progression and transformation. RAC regulates lamellae formation whereas CDC42 triggers filopodia outgrowth, and they both participate in transcriptional control via activation of JNK/stress-activated protein kinase, p38 MAPK, or nuclear factor kappa-B (Murphy et al. 1999). Their overexpression in several human tumors
make them attractive targets for cancer therapy and they are involved in a wide array of cellular functions including differentiation, survival and cell cycle progression (Fritz et al. 1999; Fritz and Kaina 2006). They are also involved in the regulation of neuronal development, survival, and death (Linseman and Loucks 2008), as well as neuronal regeneration after axotomy in mice (Tanabe et al. 2003).

The other identified binding partner of HN1 is SFPQ, also known as polypyrimidine tract-binding protein-associated splicing factor (PSF). It was first identified as a splicing factor but it also has other functions. SFPQ contains both DNA- and RNA-binding domains (Tapia-Paez et al. 2008). It is expressed in mouse germ cells and enhances androgen receptor-mediated transcription, which is important for spermatogenesis (Kuwahara et al. 2006), and it is a repressor of the progesterone receptor (Dong et al. 2005). Located on chromosome 1p34-p36, SFPQ also forms a complex that regulates the DIX1C1 gene involved in dyslexia and neuronal migration during development (Tapia-Paez et al. 2008). SFPQ seems to be involved in the control of neuronal-specific splicing events at particular stages of neuronal differentiation. During embryonic development and early postnatal life, SFPQ protein is highly expressed throughout the brain, but its expression in adult tissue is restricted to the hippocampus and olfactory bulb, and in differentiating cortical and cerebellar neurons (Chanas-Sacre et al. 1999). In zebrafish, it is most highly expressed in the brain during neuronal development. Mutations in this gene cause abnormalities in neural crest, muscle and heart development or function. Its function is required for cell survival and differentiation of some types of neurons (Lowery et al. 2007). SFPQ also appears to play a role in tumorigenesis, as it is commonly seen in papillary renal cell carcinoma. The fusion of SFPQ and the transcription factor TFE3 in healthy renal
epithelial cells leads to dedifferentiation and loss of functional proteins (Mathur and Samuels 2007). Both SFPQ and RICH2 are involved in development, neuronal regeneration and cancer.

**Hn1 Expression Paradigms**

Northern blot analyses have identified two *HNI* transcripts at 1 and 1.6 kb in human brain, placenta, testes, prostate, skeletal muscle, thymus, colon, and peripheral blood cells (Zhou et al. 2004). Murine *Hn1* encodes two mRNA species of 0.7 and 1.4 kb in hematopoietic cells, fetal liver, adult bone marrow, and spleen, as well as several cell lines, including murine erythroleukemia cells, FDC-P1 myeloid cells, EL4 T cells, 70Z/3 pre-B cells, WEHI-3 myelomonocytic cells, 3T3 fibroblasts, PCC3 embryonic stem cells and BL3 hemopoietic multipotential cells (Tang et al. 1997). Through serial analysis of gene expression (SAGE) and RT-PCR, *Hn1* expression was detected in the Ra2 immortalized microglial cell line (Inoue et al. 1999). It was also reported as one of several genes that are downregulated during ceramide-induced apoptosis of PC12 cells (Decraene et al. 2002).

When comparing the expression of genes involved in regenerating and non-regenerating models of injured motoneurons, our lab identified the upregulation of Hn1 mRNA and protein in the axotomy of the adult facial motor nerve and of the vagus nerve, both of which regenerate after injury (Zujovic et al. 2005). This phenomenon was observed in both rats and mice. By two days after axotomy of the adult facial motor nerve, Hn1 expression is upregulated in the facial motor nucleus and its expression decreases over time, returning to basal levels three to four weeks later. This temporal expression pattern correlates with the amount of time required for the injured motoneurons to regenerate. *In situ* hybridization analysis indicated that Hn1 is specifically expressed by the injured motoneurons in the facial motor nucleus. In models of neuronal injury where neurons fail to regenerate, such as the neonatal facial motor nerve axotomy and rubrospinal tractotomy, levels of Hn1 mRNA remain low and unchanged (Zujovic
et al. 2005). During the injury-induced dedifferentiation stage of Japanese common newt retinal pigment epithelial cells and in retinal progenitor cells, the ortholog of Hn1 is also upregulated (Goto et al. 2006). Because of its incidence in regenerating models, the expression of Hn1 mRNA was evaluated in developing mouse and rat tissues. Numerous tissues, including the brain, spinal cord, heart, thymus, spleen and testis, manifest high levels of Hn1 mRNA during embryonic development. The expression of Hn1 throughout the brain of the newborn rat and mouse is diffuse and intense, however in mature rodents, Hn1 expression is confined to the hippocampus, cortex, and cerebellum (Zujovic et al. 2005), areas of the brain that have relatively high plasticity and which are considered important in learning and memory. This is consistent with the identification of Hn1 mRNA expression in mice as early as embryonic day 18, and up to postnatal day 3 and 6, in corticospinal motoneurons and callosal projection neurons respectively (Arlotta et al. 2005). Another piece of evidence supporting the involvement of Hn1 in development is that Jupiter, the ortholog of Hn1 in D. melanogaster, is expressed during the development of the neural retina (Karpova et al. 2006). Moreover, Hn1 was identified by microarray analysis as one of only four genes that distinguish epithelial ovarian carcinoma cells from normal ovarian surface epithelial cells (Lu et al. 2004).

The high prevalence of Hn1 in neural regeneration, development and cancer suggest that this gene is involved in cell plasticity and growth. For many reasons, ranging from the development of anti-cancer therapies to the prevention of neurodegenerative diseases, it would be very valuable to understand the exact role of this protein. Therefore, the main focus of this investigation has been to identify the function and intracellular mechanism of Hn1. In this study, the two main models used to investigate Hn1 were the murine GL261 glioma and B16.F10 melanoma. HN1 expression was also investigated in human brain tumor tissue and several
human cancer cell lines. Lastly, because of its apparent role in plasticity and differentiation, models of differentiation and dedifferentiation were also used to investigate properties of Hn1.

**Cancer**

As is typical of all cancers, a tumor can be caused by genetic and external factors that alter the intracellular mechanisms that are responsible for controlling cell growth and repair. These alterations can prompt a single cell to begin to divide uncontrollably and invade the surrounding healthy tissue, with the possibility of metastasizing to other regions of the body. Because of its involvement in growth and plasticity-related paradigms, one factor that could potentially influence the control of cancer is Hn1.

Tumors are classified by grade based on the morphological appearance and molecular phenotype of their cells in comparison to the cells that make up the surrounding tissue. Tumors with more differentiated cells are assigned a lower grade and these are less severe than tumors that are more anaplastic and more unlike the surrounding tissue. Grades range from 1 to 4, the highest being the more dedifferentiated and usually having the worst prognosis (American Joint Committee on Cancer 2002).

**Glioma**

Tumors that form within the brain are especially challenging for oncologists to treat. Surgical removal of the tumor mass is very risky and requires invasion of the cranium and the delicate brain tissue. Also, the blood brain barrier, which filters most foreign molecules from entering the central nervous system, makes it difficult to develop chemotherapeutic agents to treat these tumors. Brain tumors are confined to the limited space within the cranial cavity, so any growth of the tumor mass, whether malignant or benign, can cause devastating effects on the cognitive, sensory or motor functions of the brain, depending on the exact location of the growing mass.
Brain tumors can be either primary intracranial tumors, which originate from any tissue within the cranial cavity, such as brain, pituitary, pineal, and meninges, or metastatic tumors which originate anywhere in the body and spread to the brain (Porth 2002). The most common primary brain tumor, and one of the most fatal and treatment-resistant of human tumors is the glioblastoma multiforme (GBM) (Henson 2006). This is a type of astrocytoma, a glioma that originates from a type of brain glial cell known as an astrocyte, and it makes up 50% of all gliomas. GBM is the most malignant form of astrocytoma (Grade 4) (Porth 2002). Each year, approximately 9000 Americans are diagnosed with GBM (Henson 2006); the average age of these patients is 64 years. Patients with this type of tumor survive only 12 to 15 months after diagnosis, on average (Henson 2006). The probability of survival after five years from the time of diagnosis is less than 4%, and the patients that do survive are almost always young (McLendon and Halperin 2003). About 5% of all glioma cases are familial and 1% of them possibly have autosomal dominant inheritance (Malmer et al. 2001). Twenty percent of brain tumors in the first two decades of life are brain stem gliomas. This type of tumor can originate as a low-risk Grade 1 astrocytoma and become highly undifferentiated and more difficult to treat over time, leading to a Grade 4 glioblastoma multiforme (Porth 2002).

The current consensus for glioblastoma treatment is maximum surgical removal of the tumor mass followed by radiation therapy in combination with chemotherapy using temozolomide, a relatively recent chemotherapeutic agent that appears to show significant prolongation of survival for patients with recurrent malignant glioma (Dehdashti et al. 2006). Administration of this drug continues for several months but glioblastomas have a high tendency to recur. When this occurs, the tumor progression is very quick and normally leads to death within several months because the migrating glioma cells become resistant to conventional
chemotherapies (Lefranc et al. 2006). Glioblastoma multiforme tissue consists of glial pleomorphism, mitotic figures, giant cells, vascular hyperplasia, and focal areas of pseudopalisading necrosis (Wu and Hsu 1994). Its diffuse characteristics make it extremely difficult to remove. In some cases, these tumors may also become radioresistant. Because the current treatments available are not very promising, there is high demand for an effective innovative treatment.

**Murine GL261 Model of Glioma**

The murine GL261 glioma model was used for the studies on the role of Hn1 in glioma growth. This model originated in 1941 after a C57BL/6 mouse was injected intracranially with 3-methylcholanthrene, which caused it to develop a tumor (Zimmerman and Arnold 1941b). The tumor was originally maintained *in vivo* by serial transplantation of small tumor pieces onto syngeneic C57BL/6 mice (Ausman et al. 1970). The GL261 permanent cell line that grows in culture *in vitro* was derived from this tumor in the 1990s (Szatmari et al. 2006). These cells have a very quick growth rate and no contact inhibition, and when injected into their syngeneic mice, a GL261 glioma grows aggressively. Intracranial implantation of 100,000 cells typically causes the mice to die within 25-30 days. The GL261 glioma model forms a much more aggressive tumor than other glioma models (San-Galli et al. 1989; Weiner et al. 1999). GL261 cells are significantly radiosensitive *in vitro*, but not *in vivo* (Szatmari et al. 2006). They can be transduced very efficiently with adenoviral vectors but not with MoMLV-derived retroviral vectors. Similar to human gliomas, GL261 tumors have elevated expression levels of mutated p53 gene, which accompanies a bad prognosis; they also share a mutation in the K-ras gene and upregulation of c-myc. A study on mice pre-vaccinated with at least one million irradiated GL261 cells 3 to 7 days prior to intracranial tumor implantation showed that the mice developed immunity to these cells and, as a result, did not develop tumors. The immunity was specific and
had long-term efficiency (Szatmari et al. 2006). GL261 tumors create a highly immunosuppressive environment; they have high levels of TGF-β and are infiltrated with microglia and lymphocytes (Liu et al. 2008). Overall, this murine model is appropriate for the study of treatments against glioma because it shares its main characteristics with human gliomas (Bos 1989; Ishii et al. 1999; Sidransky et al. 1992). They are both invasive but do not metastasize, they have high tumor take rates, mutated p53 and K-ras, and upregulated c-myc and p53 (Szatmari et al. 2006).

**Human Glioma Cell Lines**

Expression of HN1 was also evaluated in the two tumorigenic human glioblastoma cell lines U87-MG and U118-MG. These cell lines were derived from grade III glioblastomas from a 44-year-old female and a 50-year-old male respectively. Both tumors of origin were highly malignant astrocytomas. Also, mitoses occur more commonly in these cells than in cultures of normal glia. Both cell lines are hyperchromatic and sometimes grossly abnormal. Histological features of U87-MG tumors include necrosis and large tumor cells; in culture, these cells are also large and very bizarre, and they grow very slowly. The U118-MG tumor is characterized by pleomorphism and fibrosis; the cells have cytoplasmic granulation (Ponten and Macintyre 1968). Because of their human origin, their genetic alterations are representative of human glioblastomas, which makes these cells very useful in studying the molecular biology of glioblastomas.

**Melanoma**

Melanoma is the fifth and sixth most common cancer in males and females in the USA, respectively (Chiu et al. 2006) and the most frequent type of cancer in people ages 25-29. It is also one of the most common environmentally-induced cancers, due to ultraviolet radiation (Bleyer et al. 2006) from sun exposure (Ivry et al. 2006), and early detection is essential for long-
term survival (Chiu et al. 2006). Melanomas have a high tendency to metastasize and 40% to 60% of patients with metastatic melanoma are diagnosed with brain metastases. Prognosis is poor, recurrence rates are high, and average survival time is only about 6 months after diagnosis (Fidler et al. 1999). Because of its metastatic properties, melanomas are very difficult to treat. As is the case for gliomas, the combination of radiotherapy and chemotherapy is more efficient in treating melanomas than either treatment alone (Cranmer et al. 2005).

**Melanocyte Biology, Melanosome Transport and Secretion**

A melanoma likely originates when functional differentiated melanocytes undergo changes and become dedifferentiated, resembling cells in an earlier developmental stage. Melanocytes are the cells in the epidermis that produce the chemical pigment melanin, which gives color to the skin, hair and eyes. Melanocytes have melanosomes, which are vesicles that contain the melanogenic enzymes tyrosinase, tyrosinase-related protein-1 (Trp-1), and Trp-2 (also known as dopachrome tautomerase, or DCT) that synthesize melanin from L-DOPA (Lin and Fisher 2007; Yamaguchi et al. 2007). Melanosomes are transported from the perinuclear region of melanocytes to the periphery of the cell via microtubules. A protein termed Rab27a binds the melanosome and forms a complex with melanophilin and myosin Va, which then binds the peripheral actin filaments (Wu et al. 2002). This interaction of the melanosome with actin via Rab27a is necessary for the secretion of melanin onto surrounding keratinocytes, which make up the most common cell type found on the outer layer of the skin, or the epidermis. The primary function of a fully differentiated melanocyte is to synthesize and secrete melanin. When the melanocyte undergoes malignant transformation and becomes cancerous, the magnitude of these properties diminishes and the cells re-enter the cell cycle and begin to proliferate.

The regulation of melanogenesis and the cell cycle in melanoma is a complex phenomenon, controlled in large part by the microphthalmia transcription factor (MITF), which
is expressed in cells of melanocytic origin (Figure 1-6) (Carreira et al., 2005). MITF expression is positively regulated by phosphorylation at serine307 by phosphorylated p38 MAPK (Mansky et al. 2002; Saha et al. 2006) or by activation of the Wnt/β-catenin pathway (Saito et al. 2003). Conversely, phosphorylation of MITF at serine73 via phosphorylated ERK elicits transient activation of MITF followed by its proteasomal degradation (Saha et al. 2006; (Wu et al. 2000). In mouse melanoma cells, the consequences of ERK (p42/44 MAPK) activation on MITF activity are predominately inhibitory (Wellbrock et al., 2008). Besides the melanogenic enzymes, other factors that are known to be regulated by MITF include the expression of p21 and p27, c-Met, and Rab27a (Beuret et al. 2007; Carreira et al. 2005; Carreira et al. 2006; Chiaverini et al. 2008). A transcription factor related to MITF but more ubiquitously expressed is the upstream stimulating factor 1 (USF-1), which is also activated by p38 (Corre and Galibert, 2005; Corre et al., 2004). ERK-induced activation of USF-1 has also been demonstrated in arterial smooth muscle cells (Renault et al. 2005; Samarakoon and Higgins 2008). Interestingly, USF-1 is necessary for the UV-induced melanogenic response as well as for embryonic development, and it is also involved in regulating cell cycle transitions. Loss of its transcriptional activity has been detected in other cancer models including breast cancer cell lines, demonstrating its importance in the regulation of cell proliferation (Corre and Galibert, 2005).

Furthermore, the skin is not the only place where melanogenic cells can be found. They are also present, with some differences, in the retinal pigmented epithelium of the eye, in the hair cells of the inner-ear, and in the substantia nigra and locus coeruleus of the brain (Nicolaus 2005). The substantia nigra contains high levels of dopamine, whose precursor, L-DOPA, is also the precursor for melanin. The degeneration of these cells leads to Parkinson’s disease. The brains of patients with Parkinson’s disease have significantly reduced melanin in this area as
well. Newborn babies also have reduced melanin in their substantia nigra. Interestingly, these two circumstances represent inefficient or strongly compromised coordination of movement respectively (Nicolaus 2005).

**Murine B16.F10 Melanoma Model**

The melanoma model we utilized for our studies was the murine B16.F10 model. The B16 melanoma cell line was derived from a spontaneous melanoma on the ear of a C57BL/6 mouse in 1954 (Stephenson and Stephenson 1970). The B16.F10 cell line was established from the original B16 cell line by ten repetitions of *in vivo* intravenous passages into syngeneic mice and isolation of sublines *in vitro* from cells that metastasized to the lungs (Cranmer et al. 2005; Fidler 1973). For this reason, B16.F10 cells have a higher tendency than the parent B16 cells to metastasize to the lungs. This characteristic can be altered by pre-treatment with cytoskeleton-disrupting agents such as colchicine and cytochalasin B, resulting in the formation of a dorsal cerebral tumor after intravenous administration (Cranmer et al. 2005). Angiogenesis begins very quickly after establishing tumors from these cells. The expression level of albumin, an abundant protein in liver and blood, decreases with tumor progression (Culp et al. 2006). Proteins associated with electron transport, protein folding, blood coagulation and transport also decrease, whereas prohibitin, superoxide dismutase, and alpha enolase, which are proteins previously observed in cancers, increase (Culp et al. 2006). Unlike the GL261 cells, B16.F10 cells form dense tumors (Culp et al. 2006) that do not elicit a strong immune response. Several studies using the murine B16.F10 melanoma model have shown tumor regression and resistance to subsequent challenge (Heller et al. 2000; Kishida et al. 2001; Lohr et al. 2001). An effective strategy that has been used to deliver gene therapy into established B16 and B16.F10 tumors has been the use of *in vivo* electroporation using microsecond pulses (Heller et al. 2000; Kishida et al. 2001; Lohr et al. 2001). This method of gene delivery is effective and does not result in toxic
side effects (Lohr et al. 2001). A study showed B16.F10 tumor regression, resistance to distant challenge, and a survival rate of about 47% in the animals tested. This was accomplished by using gene therapy in combination with electroporation to deliver IL-12 plasmid DNA into B16.F10 tumors (Lucas et al. 2002). The therapies available to treat malignant melanomas need to be optimized for efficient tumor regression and increased survival. The B16.F10 model is an appropriate murine model to study melanomas because of its rapidly invasive and metastatic nature, which resemble characteristics of human melanomas. The high expression of Hn1 in the murine B16.F10 melanoma cells makes it a potential target for the treatment of melanoma by gene therapy.

**Human Melanoma Cell Lines**

Hn1 expression was not only investigated in the murine B16.F10 cells, but it was also evaluated in three additional melanoma cell lines of human origin, SK-mel-28, A375 and C8161. The tumorigenic human melanoma SK-MEL-28 cell line was derived from a melanoma on the skin of a 51-year-old male (ATCC, The Global Bioresource Center). A study of 35 patients from which melanoma cell lines were derived showed that this patient had the highest immune reactivity to his own cultured melanoma cells, the SK-mel-28 cells (Carey et al. 1976). The A375 malignant melanoma cell line was derived from a skin melanoma of a 54-year-old female (ATCC, The Global Bioresource Center). While these cells express intrinsic levels of the endothelin receptor type B, treatment of these cells with bromodeoxyuridine (BrdU) induces reversible differentiation, which is accompanied by induction of endothelin receptor A expression (Ohtani et al. 1997). The C8161 human melanoma cell line was derived from an abdominal wall metastasis. It is an amelanotic and highly metastatic cell line with a doubling time of approximately 6 days. In comparison to the A375 cell line, C8161 cells have 6-fold
greater c-myc expression, more than 1.5-fold less c-fos expression, and more than 2-fold greater c-jun expression (Welch et al. 1991).

**Other Human Cancer Cell Lines**

In order to expand the information about HN1 in cancer, ten additional cancer cell lines of human origin were evaluated for HN1 expression. These included esophageal squamous cell carcinoma (KYSE-140, -150, -180 and TE-1, -2, -3, -4, -5), ovarian carcinoma (OVCAR-3) and erythroleukemia (K562).

The three KYSE cell lines were derived from esophageal squamous cell carcinoma (SCC) from three different patients. KYSE-140 cells originated from a moderately differentiated SCC located in the middle intrathoracic esophagus of a 54-year-old male who had not received prior treatment for the tumor; the tumor had invaded the adventitia. KYSE-150 cells originated from a poorly differentiated SCC located in the cervical esophagus of a 49-year-old female who had received irradiation as treatment; the tumor had invaded contiguous structures. KYSE-180 cells originated from a well-differentiated SCC located in the middle intrathoracic esophagus of a 53-year-old male who had not received prior treatment for the tumor; the tumor had invaded the adventitia. At 27.4 hours, the doubling time for the moderately differentiated KYSE-140 is approximately twice as long as that for the well-differentiated KYSE-180 and the poorly differentiated KYSE-150. The KYSE-140 cells are also less efficient at forming colonies on plastic than the other two cell lines (Shimada et al. 1992).

The TE cell lines were also derived from human esophageal SCCs. TE-1, -2, and -5 were derived from primary tumors and TE-3 and -4 were derived from metastatic tumors (Nakajima et al. 2004). TE-1 cells were derived from a well-differentiated tumor. Their doubling time is 60 hours and they have distinctive marker chromosomes, a male karyotype and abundant organelles. TE-2 cells were derived from a poorly differentiated tumor. Their doubling time is 72 hours and
they lack marker chromosomes; they also have very few organelles. Both cell lines have desmosomes and interdigitated microvilli and both are tumorigenic (Nishihira et al. 1979). The epithelial cell line TE-3 was derived from a metastatic lesion on a patient’s right chest wall, which originated from a well-differentiated SCC of the esophagus. Its doubling time (48 h) is shorter than that of TE-1 and -2. They have distinctive marker chromosomes, a male karyotype, desmosomes and microvilli, and are rich in cell organelles (Kuriya et al. 1983). TE-4 cells were derived from a metastatic well-differentiated esophageal SCC (Nakajima et al. 2004). TE-3 and -4 cells are sensitive to tumor necrosis factor (TNF) whereas TE-1, -2 and -5 are not. However, TE-3 and -2 are the only ones sensitive to interferon gamma (IFN-gamma), an inducer of TNF receptor (Suzuki et al. 1989). TE-5 cells were derived from a poorly differentiated tumor. A more recent investigation reports very different doubling times for all these cell lines; approximately 25, 14, 17, 28 and 24 hours for the TE-1, -2, -3, -4 and -5 cells, respectively (Zhao et al. 2000).

The OVCAR-3 cell line was derived from the poorly differentiated malignant ascites of a patient with progressive adenocarcinoma of the ovary after combination chemotherapy with cyclophosphamide, adriamycin and cisplatin. These cells are tumorigenic, clonogenic in agarose, and have an abnormal karyotype. They also contain androgen- and estrogen-receptor-like macromolecules in their cytoplasm. The cell line continues to have the characteristics of the tumor from which it originated and is therefore an excellent model to study the molecular basis of human ovarian carcinoma (Hamilton et al. 1983).

The K562 cell line originated from a 53-year-old female chronic myeloid leukemia patient in terminal phase blast crisis (Klein et al. 1976). It is an undifferentiated blast cell and contains no granules or lymphocyte markers. These cells grow in suspension culture and their average
doubling time is 12 hours. They synthesize glycophorin A and are therefore considered very early erythroid precursors (Koeffler and Golde 1980). The transcription factors MITF, USF-1, and USF-2 are expressed in the nuclei of K562 cells and bind the HOX response element (HXRE-2) site of homeobox B4 (HOX4B). However, only USF-1 and -2 are able to upregulate HOX4B transcription (Giannola et al. 2000). Like HN1, HOXB4 is located in chromosome 17, locus 17q21-q22 (NCBI Entrez Gene). An important transcription factor for development, HOXB4 regulates the balance between hematopoietic stem cell renewal and differentiation. It is highly expressed in primitive cells and declines with terminal differentiation (Giannola et al. 2000).

Detection of HN1 in one or more of these human cancer cell lines would validate the significance of the experiments conducted in the murine glioma and melanoma models, since Hn1 would be identified as a gene that is present in more than one type of human cancer and may therefore have a role in cancer biology. Moreover, evaluation of HN1 expression in relation to the numerous characteristics provided by this variety of cell lines may provide insight into its function.

**Differentiation**

In development, cells proliferate, then they migrate to their target locations and finally, they begin to differentiate into specific cells with specific functions. Cell differentiation is normally characterized by cell cycle arrest, expression of specific cell markers, and full functionality of the specific cell type. Varying stages of differentiation are possible throughout the development of an organism. Usually, cancer cells and cells with high plasticity or undergoing post-injury regeneration are considered to be temporarily less differentiated than the adjacent non-transformed cells. Hn1 is highly expressed in multiple tissues during rodent embryonic development and in plastic regions of the adult rodent brain (Zujovic et al. 2005). Its
expression is also highly upregulated during the neural regenerative period after adult rodent facial nerve axotomy and vagotomy, as well as in the dedifferentiated retinal pigment epithelial cells during regeneration of the Japanese common newt retina (Goto et al. 2006; Zujovic et al. 2005). Moreover, Hn1 is one of only four genes that distinguish epithelial ovarian carcinoma cells from normal ovarian surface epithelial cells (Lu et al. 2004). The prevalence of Hn1 in development, regeneration, plasticity and cancer suggests the possibility that it may have a role in the regulation of cell differentiation. To test this general hypothesis, a series of experiments have been designed to evaluate the role of Hn1 in cell differentiation using two cell culture models, namely the RAW264.7 and HEK293 cells.

**RAW264.7 Cells**

The transformed murine macrophage cell line, RAW264.7, was established from a tumor induced by Abelson murine leukemia virus (ATCC, The Global Bioresource Center). This cell line expresses high levels of the receptor activator of nuclear factor kappa B (RANK), which is expressed by the precursors of osteoclasts. Osteoclasts are the multinucleate cells responsible for bone resorption. Differentiation of RAW264.7 cells into osteoclasts is induced by stimulation with the RANK ligand (RANKL), a member of the TNF family (Hsu et al. 1999). The differentiation of RAW264.7 cells into osteoclasts by RANKL treatment is positively regulated by p38 MAPK and negatively regulated by the MEK/ERK MAPK pathway (Hotokezaka et al. 2002). The density of the cells at the time of plating is critical for RANKL-induced differentiation of these cells (Hayashi et al. 2008; Hotokezaka et al. 2002). Multiple transcription factors are involved in driving the differentiation of these cells. Among them are MITF and PU.1. There is also evidence that TFE3 is involved in this pathway because mice with a null allele of TFE3 have severe osteoclast abnormalities (Sharma et al. 2007).
HEK293 Cells

HEK293 cells were derived from primary human embryonic kidney (HEK) cells that were transformed with sheared adenovirus-5 (Graham et al. 1977). Characterization of these cells indicates that their protein expression pattern resembles that of neuronal cells more than that of kidney cells. They express intermediate filaments including the neurofilament proteins NF-L, NF-M and NF-H, α-internexin, and vimentin, as well as certain neurotransmitters, neurotransmitter receptors, ion channels and other neuron-specific markers. Because they express high levels of NF-M, NF-L and vimentin, and low levels of NF-H and α-internexin, they are said to resemble early differentiating neurons (Shaw et al. 2002). The expression patterns of these neurofilaments serve as indicators for different stages of neuronal differentiation, because developmentally, α-internexin expression precedes NF-L and NF-M expression (McGraw et al. 2002; Shaw et al. 2002). NF-H expression appears last, and some fully differentiated neurons never express it (McGraw et al. 2002; Shaw et al. 2002).

Significance and Specific Aims

The hematopoietic and neurologic-expressed sequence 1 (Hn1) encodes a protein of unique amino acid sequence whose function is yet unknown. There are multiple reasons why it is significant to study the function and mechanism of Hn1 protein. First, the potentially important role that this protein plays in the rapid growth of malignant tumors such as gliomas and melanomas could make it a promising target for anti-cancer therapies. Second, the elevated expression of Hn1 during regeneration of injured neural tissue and brain plasticity make it an attractive target for the potential prevention and healing of motoneuron degenerative diseases such as Amyotrophic Lateral Sclerosis, Parkinson’s and Alzheimer’s diseases, and it could provide insight into the molecular mechanisms underlying learning and memory processing.
Third, because it is expressed in multiple tissues during embryonic development and may have a critical role in cell differentiation, Hn1 is a promising gene to study. The GL261 and B16.F10 models of murine cancer serve as useful tools to study aspects of the intracellular function of Hn1.

Like many forms of cancer, both gliomas and melanomas are comprised of cells that have high proliferative capacity and display gene expression patterns and behaviors consistent with progenitor cells. Thus, clues to the biological mechanisms of these cancers will likely come from the characterization of genes involved in development and growth. The primary goal of this project was to identify the function(s) of Hn1 in glioma and melanoma cells. The main hypothesis was that Hn1 expression plays an important role in regulating the growth of gliomas and melanomas in vivo. The specific aims of this study were designed to:

1. Identify the in vitro and in vivo effects of Hn1 in murine glioma GL261 cells. The first objective was to identify Hn1 protein expression in GL261 cells and determine its cellular localization by immunohistochemical analysis of cultured cells and tissue sections derived from intracranial GL261 tumors. The second objective was to compare the effect(s) on the growth rates of cells with and without Hn1 in vitro and the in vivo growth of tumors established from GL261 cells with and without Hn1. For mechanistic insight, the third objective was to identify the proteins that bind to Hn1 through immunoprecipitation analyses.

2. Determine the intracellular mechanism of Hn1 in the murine B16.F10 melanoma cell line cultured in vitro. The first objective was to identify Hn1 protein expression and localization in these cells. The second objective was to compare the in vitro growth rates of B16.F10 cells with and without Hn1. The third objective was focused on evaluating the intracellular effects of Hn1 protein expression in these cells.
3. Evaluate HN1 expression in human cancers. The objective was to identify the levels of HN1 expression in human brain tumor tissues and different human cancer cell types, and to assess whether the levels of HN1 expression correlated with any of their particular cellular characteristics.

4. Investigate the role of Hn1 in cell differentiation. The first objective was to evaluate the effect of RANKL-induced RAW264.7 cell differentiation on Hn1 expression as well as the effect of Hn1 expression on RAW264.7 cell differentiation independent of RANKL stimulation. The second objective was to identify the effects of HN1 overexpression on the expression of neurofilament proteins in HEK293 cells and gain insight on their resulting differentiation state.
Figure 1-1. Sequence alignment of human, murine and rat Hn1. Identical amino acids are indicated by vertical lines. Non-identical, functionally conserved amino acids are indicated by colons. The three orange boxes indicate consensus sites for N-linked glycosylation, and the blue box indicates the SUMOylation consensus site. PPGG and MASNIF motifs are underlined.
| Human HN1 | MTTTTTFKGVD.PNSRNSSRVLRRPPCGGSNSLGFDEPTEQPVKRNKMASNIF |
| Human HN1L | MFQVDPSEGGRAGSRAMKPPGGESSNLFGSPEEATPSSRPNRMASNIF |
| Jupiter | MISNFDCDNQASSKVRPPGGGSDFGSEMPQTPRNVKRNRMASNIF |

| GTPEE...WQASWAKSAGAKSSGREDLESSGLQRNSSEASSGDFLDLKGEGD......IHENVD |
| GPTEEPQNIKPRTNPPGGKGSIFD..ESTPVTQRQLNPPTGKTSIPFGSPVTATSRHLAHPPK |
| AA..EKNVGNKNDAPPRGQKTVDSHSLFGEPTRPITPGLKNHMKSSIPFGQNTVEAAQKLLT |

| TD.................PGSLGQS......EEKPVPPA.PVPSVAPAP.V. |
| KD.HVFLCEGEEPKSDLKAARSHAPAGAEPKGEKSGARKAPRAEQEPMPT.VDSHEPRLGPRPRSH |
| TNGHYNGKSGSVSSASSVSSTENLKMSGSRSSEGPNVTGEGYKVANEYSSQRQESSNGGPVI |

| PSRRNPPGGKSSLVLG |
| NKVLPFGGKSSISFY |
| NKNRPFGGYSSGLW |

Figure 1-2. Sequence alignment of the human HN1 and HN1-like (HN1L) with *D. melanogaster* Jupiter. Vertical lines indicate identical amino acids. Red amino acids are conserved in all three sequences. The PPGG and MASNIF motifs are underlined.
Figure 1-3. Ratio of the incidence of each amino acid in Hn1 compared to the average protein. The incidence of each amino acid in an average protein is shown in purple while the incidence in Hn1 is shown in red. There is high incidence of small turn-promoting amino acids (proline, serine, glycine) and low incidence of hydrophobic amino acids (glutamine, histidine, tyrosine, tryptophan) and cysteine, methionine, cysteine, phenylalanine, leucine, and isoleucine in Hn1 compared to the average protein.
Figure 1-4. Phosphorylation sites on human HN1. The phosphorylated amino acids identified by Dephoure et al. (Dephoure et al. 2008) are shown in bold. Underlined is the motif surrounding the phosphorylation of S71, which matches the phosphorylation consensus site identified in multiple proteins during mitosis.
Figure 1-5. Predicted structure model of murine Hn1. This was obtained by PyMol software using the amino acid sequence coordinates predicted by the HHMSTR/ Rosetta program on the ExPASy portal. A) Cartoon model showing four possible small α-helices (red) and two β-sheets (yellow). B) Space-filling model of the predicted structure of this molecule.
Figure 1-6. Diagram of the complex regulation of MITF in pigment cell and melanoma proliferation and differentiation. The pathway was derived from a modification of diagrams in other publications (Busca and Ballotti 2000; Carreira et al. 2005).
CHAPTER 2
METHODS

Cell Culture

GL261 (NCI, Frederick, MD), K562 cells (ATCC, Manassas, VA), C8161 (kindly provided by Dr. Bernard Weissman, University of North Carolina, Chapel Hill, NC), KYSE 140, 150 and 180 (from Dr. Ishiguro at Nagoya City University, Japan), and TE 1-5 (from Dr. David Sidransky at Johns Hopkins University, Baltimore, MD) were grown in RPMI 1640 (Gibco BRL, Grand Island, NY) containing 10% FBS and 1% penicillin-streptomycin (1000 units/ml of penicillin, 1000 µg/ml or streptomycin) (Gibco BRL, Grand Island, NY). RPMI for GL261 cells was supplemented with 4mM L-glutamine. B16.F10, HEK293, RAW264.7, SK-mel-28, NMuMG, OVCAR-3, A375, U118-MG and U87-MG were obtained from ATCC (Manassas, VA). A375 cells were grown in DMEM containing 10% FBS and 1% penicillin-streptomycin. B16.F10, HEK293, and RAW264.7 cells were grown in DMEM (Gibco BRL, Grand Island, NY) containing 10% FBS, 1% penicillin-streptomycin, and 1% sodium pyruvate (0.11 mg/ml) (Sigma-Aldrich, St. Louis, MO). The sodium bicarbonate content in DMEM was 3.7 g/L for HEK293 and RAW264.7 cells, and 1.5 g/L for B16.F10 cells. U118-MG cells were grown in DMEM with 4 mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose and 10% FBS. NMuMG and OVCAR-3 cells were grown in DMEM with 10% FBS (heat-inactivated). U87-MG cells were grown in Minimum essential medium (Gibco BRL, Grand Island, NY) with 2 mM L-glutamine and Earle's BSS adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, and 10% FBS. SK-mel-28 cells were also grown in Minimum essential medium supplemented with L-glutamine and Earle's BSS, 10% FBS, 1% penicillin-streptomycin, and 1% sodium pyruvate. Trypsin-EDTA was used to subculture all of the cells, except the K562 and RAW264.7 cells, which were subcultured either
by suspension or gentle scraping respectively. All cells were cultured in an incubator maintained at 37°C with 5% CO₂.

**Northern Blot Analysis**

Total RNA was isolated from GL261, B16.F10, K562 and OVCAR3 cells using TRIzol Reagent according to the manufacturer's recommended procedure (Life Technologies, Grand Island, NY). RNA (20 μg/lane) was electrophoresed through denaturing 1.2% agarose and subjected to Northern blot analysis (Church and Gilbert 1984). The nylon membrane was hybridized with a ³²P-radiolabeled cDNA generated from a 450-bp murine Hn1 protein coding DNA sequence, which was ³²P-radiolabeled by the random primer method to a specific activity of 1.2×10⁹ dpm/μg.

**Development of Anti-Hn1 Antibodies**

Mouse Hn1 was expressed in *E. coli* using the pET22a and pATH11 expression vectors. The pATH11 vector produces Hn1 fused to the C-terminus of *E. coli* Trp-E, while pET22a produces a His-tagged protein. The Trp-E fusion protein was purified by excision of the appropriate gel band from a 6 M urea extract of a bacterial inclusion body preparation as described (Harris et al., 1991). The His-tagged protein was affinity purified using a nickel-chelating column chromatography. Mice (Balb/c) and rabbits (New Zealand white) were injected with the Trp-E-Hn1 fusion protein and then boosted with His-tagged Hn1. Sera were tested by Western blotting for activity on Trp-E-Hn1 and His-tagged Hn1. Strong reactivity with both proteins indicated good reactivity with Hn1. Serum was collected from rabbits and affinity purified on His-tagged Hn1 coupled to cyanogen bromide activated Sepharose 4B. Spleen cells from a mouse with a strong Hn1-specific immune response were fused to the SP0 myeloma line to produce hybridoma cells, which were screened initially by ELISA on His-tagged Hn1, and
then by Western blotting and immunocytochemistry. Two clones, 3C4 and 3G6, were selected for further study. Both are IgG2a class with kappa light chains and appeared to be similar or identical in their properties. Clone 3C4 was used in the present studies.

Western Blot Analysis

Protein lysates for Western blot analysis were prepared by either of two methods. For the first method, cells on a 24- or 12-well dish were rinsed with room temperature PBS and collected with 50 or 100 µl respectively of 1X SDS sample buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 0.1% Bromphenol blue, 10% glycerol, and water), sonicated on ice, supplemented with 3% β-mercaptoethanol, and boiled for 5 minutes. The second method was used for experiments where the phosphorylation state of proteins was analyzed. Cells on a 24-well dish were rinsed with ice-cold PBS and covered with 50 µl ice-cold lysis buffer (1% NP-40, 0.5% Na-deoxycholate, 0.1% SDS, and PBS) containing 10% of a protease and phosphatase inhibitor cocktail (1 mg/ml PMSF, 0.2 mg/ml aprotinin, 1 mM DTT, 1.84 mg/ml Na-vanadate, 69% isopropanol) for 5 minutes on ice. Cells were scraped from the dishes and collected for sonication and centrifugation at 4°C for 10 minutes at 16,000 x g. The supernatant was transferred to a new tube, supplemented with 4X SDS sample buffer and 3% β-mercaptoethanol and boiled for 5 minutes.

Protein samples were subjected to SDS-PAGE, transferred onto PVDF membranes and probed with corresponding antibodies. The murine monoclonal anti-Hn1 antibody was used at 1:500 and the rabbit polyclonal anti-Hn1 antibody at 1:1000. Both antibodies were developed by Dr. Gerry Shaw at EnCor Biotechnology, Inc. in Gainesville, FL. The antibodies for tyrosinase (Pep7), Trp1 (Pep1), and Trp2 (Pep8), from Dr. Vincent Hearing, National Institutes of Health, were each used at 1:2000. The monoclonal anti-Rab27a antibody from Drs. Miguel Seabra and Alistair Hume, Imperial College London, was used at 1:1000. The mouse monoclonal anti-actin...
and rabbit polyclonal anti-β-catenin antibodies (Sigma-Aldrich, St. Louis, MO) were used at 1:1000 and 1:8000 respectively. Antibodies for p21 (1:1000), p27 (1:600), cyclins A and E (1:1000), and USF-1 (1:200) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The cyclin D1 antibody (1:1000) was purchased from Neomarkers (Lab Vision Corporation, Fremont, CA) and the antibodies for TFE3 (1:500) and total Rb (1:1000) were purchased from Pharmingen (BD Biosciences, San Jose, CA). The antibodies for the various phosphorylated forms of Rb, total and phosphorylated p38, total and phosphorylated ERK, total and phosphorylated Akt, and c-Met (all used at 1:1000) were purchased from Cell Signaling Technology (Danvers, MA). The C5 monoclonal antibody for MITF (Abcam Inc., Cambridge, MA) was used at 1:2000. The secondary antibodies used were either HRP-conjugated sheep anti-mouse from Sigma-Aldrich or goat anti-rabbit from Cell Signaling Technology.

**Development of siRNA Against Hn1**

Using siDirect (http://design.RNAi.jp), three small interfering RNA (siRNA) sequences were identified that predicted maximum Hn1-target specificity to degrade the murine Hn1 mRNA. These siRNA sequences were initially tested for their ability to inhibit expression of murine Hn1 in a co-transfection paradigm using HEK293 cells, which lack endogenous Hn1. The sequences were individually cloned into a vector (pH1rSC) containing the H1 promoter that allows for packaging into recombinant self-complementary (double stranded) adeno-associated virus (AAV) (McCarty et al., 2001). The most efficient siRNA-expressing plasmid (Hn1-siRNA) was generated by cloning the following complimentary oligonucleotides into the Ascl and NheI sites of pH1rSC: 5’-CGC GGG GAG AAG GTG ATA TGC ATT TCA AGA CAA TGC ATA TCA CCT TCT CCC TTT TTG GAA A-3’ and 5’-CTA GTT TCC AAA AAG GGA GAA GGT GAT ATG CAT TGT CTT GAA ATG CAT ATC ACC TTC TCC C-3’. A second highly
efficient Hn1-specific siRNA (Hn1-siRNA-B) was also developed and used to determine if off-target effects were associated with the Hn1-siRNA described above. The sequences of the two complimentary oligonucleotides used to generate siRNA-B were: 5’-CGC GCT GTG AGG AAG AAC AAG ATT TCA AGA CAA TCT TGT TCT TCC TCA CAG TTT TTG GAA A-3’ and 5’-CTA GTT TCC AAA AAC TGT GAG GAA GAA CAA GAT TGT CTT GAA ATC TTG TTC TCC CTC ACA G-3’. A scrambled-siRNA was also developed for use as a control for the impact of expressing a double-stranded hairpin siRNA. The sequences of the two oligonucleotides used to generate the scrambled siRNA were: 5’-CGC GGG TCG AAT TCG ATA TCC TAT TCA AGA CAT AGG ATA TCG AAT TCG ACC TTT TTG GAA A-3’ and 5’-CTA GTT TCC AAA AAG GTC GAA TTC GAT ATC CTA TGT CTT GAA TAG GAT ATC GAA TTC GAC C-3’. The three plasmids were packaged into AAV6, a serotype of AAV that was determined to be the most efficient at transducing GL261 and B16.F10 cells in culture by fluorescence microscopy of GL261 cells transduced with the various GFP-expressing AAV serotypes 1-9 (Figure 2-1). Additionally, Hn1-siRNA-B was packaged into AAV2 because of its ability to transduce other cell types and this virus was diluted in iodixanol. High titer virus was produced and purified according to previously published protocols (Zolotukhin et al., 2002).

GL261 cells were seeded on 12-well cell culture plates at a density of 300,000 cells per well. Two days later, cells were rinsed with serum-free RPMI and transduced with serum-free media containing either the Hn1-siRNA-AAV6 or the control H1-AAV6. All dishes were incubated for 3 hr at 37°C in 5% CO₂, after which the volumes were doubled with media containing 20% FBS, 1% penicillin-streptomycin, and 4mM L-glutamine. Two days after transduction, protein extracts were analyzed by Western blot analysis.
B16.F10 cells were seeded on 24- or 6-well cell culture plates. One day later, representative wells of cells were counted and cells in the remaining wells transduced at a multiplicity of infection (M.O.I.) of 5000, unless otherwise indicated, with serum-free media containing AAV6 expressing Hn1-siRNA, scrambled-siRNA, control-AAV6 (containing the H1 vector alone), or no virus. All dishes were incubated for three hours at 37°C in 5% CO₂, after which the volumes were doubled with media containing 20% FBS, 1% penicillin-streptomycin and 1% sodium pyruvate. The viral vector transduction was repeated one day later unless otherwise indicated. Western blot, cell cycle, and immunocytochemical analyses were carried out two or three days after the first viral transduction. For evaluating a potential non-specific impact of the viral transduction on melanin production, the control virus was also used at 30,000 M.O.I.

RAW264.7 cells were seeded at 10,000 cells per well on a 24-well dish. The next day, cells were transduced at an M.O.I. of 5000 with Hn1-siRNA-B AAV2 and scrambled-siRNA AAV6 under serum free conditions and supplemented for a 10% total serum content three hours later.

**GL261 and B16.F10 Tumor Preparations**

GL261 cells were harvested from plates by trypsinization, washed one time in phosphate buffered saline (PBS), and suspended in PBS at a density of 67,000 cells/µl. Cells (200,000) were implanted intracranially into isoflurane-anesthetized female C57BL/6 mice (seven weeks old) on the right hemisphere of the brain (1 mm posterior and 2 mm lateral to Bregma; 3 mm deep to the dural surface). The mice were monitored carefully for signs of depressed activity and motor instability. Twenty days after GL261 cell implantation, the mice were given an injection of sodium pentobarbital (32 mg/kg) and euthanized by transcardial perfusion with 0.9% saline followed by buffered 4% paraformaldehyde (PFA). The brains were removed, post-fixed for 1 h
with 4% PFA, sectioned, cryoprotected overnight with 30% sucrose, and frozen in isopentane cooled by liquid \( N_2 \). Coronal cryosections (20 \( \mu \text{m} \)) of the brainstem and midbrain were prepared at the level of the tumor. Sections were thaw mounted on Superfrost/Plus slides (Fisher Scientific, Pittsburgh, PA) and subjected to \textit{in situ} hybridization or immunohistochemical procedures.

For experiments designed to evaluate the effect of Hn1 depletion on the growth of GL261 tumors, GL261 cells were initially seeded at a density of approximately \( 2 \times 10^6 \) cells per 100 mm plate. Two days later, cells were transduced at an M.O.I. of 5000 with either the siRNA-AAV6 or the control H1-AAV6 vector under serum-free conditions. Three hours later, the cells were supplemented with medium containing serum to achieve a final 10% FBS content. Two days after transduction, cells were prepared for intracranial implantation as described above.

B16.F10 cells were harvested from 100 mm plates by trypsinization, washed one time in sterile phosphate buffered saline (sPBS), and finally suspended in sPBS. Cells (300,000 in 9 \( \mu \text{l} \)) were implanted subcutaneously into isoflurane-anesthetized female C57BL/6 mice (ages six to eight weeks). Two weeks after cell implantation, mice were given an injection of sodium pentobarbital (32 mg/kg) and euthanized by transcardial perfusion with 0.9% saline followed by buffered 4% paraformaldehyde (PFA). Skin tumors were removed, post-fixed for 1 h with 4% PFA, sectioned, cryoprotected overnight with 30% sucrose, and frozen in isopentane cooled by liquid \( N_2 \). Cryosections (20 \( \mu \text{m} \)) of the tumor were prepared and thaw-mounted on Superfrost/Plus slides (Fisher Scientific, Pittsburgh, PA) and finally subjected to either \textit{in situ} hybridization or immunohistochemical procedures.

**In Situ Hybridization Analysis**

Hn1 hybridization probes (Zujovic et al. 2005) were used for \textit{in situ} hybridization (ISH) analysis as previously described (Harrison et al. 2003). Sections were hybridized separately with
antisense and sense riboprobes and then apposed to film. Subsequently, the sections were dipped in LM-1 emulsion. After exposure for 1 week, slides were developed, fixed and counterstained with hematoxylin and eosin (H&E).

**Immunohistochemistry**

Tissue sections (20 μm) from PFA-fixed murine GL261 tumor-bearing brains were permeabilized using 0.5% Triton X-100 for 10 min and blocked with 3% BSA for 20 min. The rabbit polyclonal anti-murine Hn1 primary antibody was used at 1:50 dilution in PBS for 1 h at room temperature. Sections were rinsed in PBS three times for 10 min and an anti-mouse Alexa Fluor 594 red fluorescent secondary antibody (Sigma-Aldrich) was added at 1:2000 in PBS for 1 h at room temperature.

Human brain tumor tissue microarray (TMA) slides obtained from the University of Florida, Department of Pathology, Immunology, and Laboratory Medicine were constructed as previously reported (Qiu et al. 2008) The final TMA consisted of 28 gliomas, including samples of WHO Grade IV (glioblastoma multiforme) and WHO grade III (anaplastic astrocytoma, anaplastic oligodendroglioma, anaplastic oligoastrocytoma), and 7 non-neoplastic control brain samples. Immunohistochemical analysis was performed by incubating with the rabbit polyclonal anti-Hn1 antibody (1:200) overnight. This was followed by incubation with a biotinylated secondary antibody (1:500) and with HRP-conjugated avidin (1:500) (DakoCytomation, Copenhagen, Denmark). The bound antibodies were visualized with avidin-biotinylated peroxidase complex and diaminobenzidine tetrachloride.

Tissue sections of subcutaneous B16.F10 tumors were permeabilized using 0.5% Triton X-100 for 10 min and blocked with 3% BSA for 20 min. The affinity purified polyclonal anti-Hn1 antibody was used at 1:50 in PBS for 1 h at room temperature. Sections were rinsed in PBS three times for 10 minutes and an anti-mouse Alexa Fluor 594 red fluorescent secondary antibody
(Sigma-Aldrich, St. Louis, MO) was added at 1:2000 in PBS for 1 h at room temperature. The sections were counterstained with DAPI at 1:333 in PBS.

B16.F10 cells cultured in vitro were plated on glass coverslips in 24-well cell culture dishes and transduced as described above. Three days after the first viral transduction, cells were fixed with 4% PFA for 30 minutes at 4°C and stained. The antibodies used were the polyclonal anti-Hn1 (undiluted), Pep7 (Tyrosinase), Pep1 (Trp1) and Pep8 (Trp2), all at 1:200, polyclonal anti-Rab27a (1:100), and phalloidin-488 (Invitrogen, San Francisco, CA) at 1:40. Immunofluorescence was analyzed using a confocal microscope (Leica, Microsystems Heidelberg GmbH, Version 2.61, Build 1538).

**Immunoprecipitation Analysis**

Cells (GL261 or B16.F10) were plated on two 150 mm dishes and allowed to grow to full confluency, at which time the cells were rinsed with cold PBS and the cell lysate was collected using 1.125 ml per dish of a buffer containing 20 mM Hepes, pH 7.6, 5% glycerol, 0.1% Triton X-100, 0.1% β-Mercaptoethanol, 1 mM EDTA, 1 mM EGTA, 20 mM tetrasodium pyrophosphate, 1 mM Na3VO4, 145 mM KCl, supplemented with protease inhibitors. The cell lysate was collected, subjected to sonication on ice, and subsequently centrifuged at 16,000g for 5 min at 4°C. The supernatants were precleared by incubating on a shaker overnight at 4°C with 50 µl recombinant Protein G Sepharose beads (Invitrogen, San Francisco, CA). The tubes were subjected to centrifugation at 800 x g at 4°C for 5 min. The remaining supernatants were centrifuged again 16,000 x g for 1 min at room temperature. The pre-cleared lysates were aliquoted into equal parts of 2.2 mg protein each. They were then supplemented with one percent (mass of antibody/mass of lysate protein) polyclonal rabbit anti-Hn1 antibody (Sigma-Aldrich, St. Louis, MO) or PBS. In a separate immunoprecipitate reaction, the pre-cleared lysate was
supplemented with either polyclonal rabbit anti-actin antibody or non-immune rabbit IgG. In each case, these were incubated on a shaker for 2 h at room temperature. Recombinant Protein G Sepharose beads (50 µl) were then added and the reactions were incubated on a shaker overnight at 4ºC. The beads in each tube were subjected to five washes, consisting of centrifugation at 16,000 x g for 1 min at room temperature, aspiration of the supernatant, and re-suspension of the pellet in 1 ml Tris buffered saline with 0.1% Tween (TBS-T). The final pellets were resuspended in 50 µl of SDS sample buffer and subjected to SDS-PAGE followed by either silver stain, mass spectrometry or Western blot analyses.

For the actin-Rab27a co-immunoprecipitation analysis, B16.F10 cells plated on two 150 mm dishes were transduced twice with either Hn1- or scrambled-siRNA AAV6. Two days later, cells were collected and processed as described above using the anti-actin or non-immune rabbit IgGs. The final pellets were resuspended in 80 µl of SDS sample buffer and subjected to SDS-PAGE followed by Western blot analysis using the monoclonal murine anti-actin and anti-Rab27a antibodies.

For silver stain analysis, the SDS-PAGE gel containing GL261 samples was fixed overnight in an aqueous fixing solution composed of 50% methanol, 12% acetic acid, and 0.37% formaldehyde. The gel was then washed with 50% ethanol three times for 20 min each. This was followed by pre-treating the gel with 0.8 mM sodium thiosulfate pentahydrate in water for exactly 1 min and washed three times with water for exactly 20 min each. The gel was impregnated with 11.8 mM silver nitrate and 0.028% formaldehyde in water for 20 min, and then washed twice with water for exactly 20 seconds. The gel was developed by submerging it in an aqueous solution made up of 0.019% formaldehyde, 566 mM sodium carbonate, and 0.016 mM sodium thiosulfate pentahydrate. It was washed twice with water for 2 min and the reaction was
stopped by incubating with an aqueous solution of 50% methanol and 12% acetic acid for 10 min. This was washed with 50% methanol for at least 20 min and stored at 4°C.

For mass spectrometry analysis, an unstained gel containing GL261 samples was incubated in colloidal Coomassie solution overnight at room temperature. Excised bands of proteins more prominent in the immunoprecipitation reactions containing the anti-Hn1 antibody were submitted to the Proteomics Laboratory at the ICBR, University of Florida and subjected to mass spectrometry analysis.

**In Vitro Cell Proliferation Assay**

GL261 cells were seeded on six-well dishes at a density of 10,000 cells per well. Two days later, cells were transduced at an M.O.I. of 5000 with siRNA-AAV6, H1-AAV6 control or no virus. Two days after transduction, triplicate wells of cells were collected by trypsinization and counted manually using a hemacytometer. This procedure was repeated every 24 or 48 h.

B16.F10 cells were seeded on six-well dishes at a density of 10,000 cells per well. The following day, cells were transduced at an M.O.I. of 5000 with Hn1-siRNA-AAV6, control H1-AAV6, or no virus. Two days after transduction, triplicate wells of cells were collected by trypsinization and counted manually using a hemacytometer. Cell counts were obtained every 24 or 48 h.

**Cell Cycle Analysis**

Cells were plated on 6-well dishes and treated with AAV6 expressing either scrambled or Hn1-siRNA or no virus. Two and three days later, cells (approximately 75% confluent) were trypsinized and collected in a 1.5 ml tube using DMEM with serum. An aliquot was used to count cells manually using a hemacytometer. The remaining cells were pelleted by centrifugation at 1000 x g for 5 minutes and the medium was aspirated carefully. For 300,000 to 500,000 cells, 500 µl of propidium iodide solution (containing 25 µg propidium iodide, 0.5 mg sodium citrate,
0.5 µl Triton X-100, 5 µg/ml RNAse A, and water) was added and slowly pipetted up and down ten times. Tubes were incubated at room temperature in the dark for two hours. Cells were transferred to polystyrene round-bottom tubes (Falcon) on ice and analyzed by Flow cytometry on a FACSsort machine.

**Tumor Volume Calculation**

Serial sections of each tumor were collected. Tumor volumes were calculated by measuring the longest and shortest diameters on each section, using the formula: Area = \((r_1)(r_2)\pi\) and then multiplying each area by the width of the corresponding section. The volumes from all the sections pertaining to a single tumor were summed to obtain the final tumor volume. Paired student’s t-test was used for statistical analysis.

**Quantification of Melanin**

B16.F10 cells were plated on 24-well dishes and transduced the next day with either scrambled- or Hn1-siRNA AAV6 at an M.O.I. of 5000 (9 wells of each) as indicated above or no virus (21 wells). Starting two days later, and daily for the next three consecutive days, media (600 µl) from triplicate wells of cells from each treatment were collected separately into 1.5 ml tubes. The remaining cells were counted using a hemacytometer to determine the number of cells per well. PBS (400 µl) was added to each tube containing medium for a total volume of 1ml. The solution was made 0.8 mM in NaOH by the addition of 0.8 µl of 1M NaOH. Floating cells in the media were pelleted and discarded by spinning at 25°C for 10 minutes at 800 x g. Absorbance measurements (500 nm) were made and the content of melanin was calculated by comparison to a melanin standard curve (0 to 40 µg) generated by adding synthetic melanin (Sigma-Aldrich, St. Louis, MO) to tubes containing media from 4 wells of cells that were not treated with virus.
determine the amount of melanin per cell, melanin content was divided by cell number in each well.

**HGF Stimulation of Phosphorylated-ERK (p42/44 MAPK)**

B16.F10 cells were plated on 12-well dishes and transduced the next day, at an M.O.I. of 3000, with either the control-AAV6 or the Hn1-siRNA AAV6. The following day, the cells were serum-starved for approximately 16 h, after which time they were stimulated with murine hepatocyte growth factor (HGF) at concentrations of 0, 10, or 100 ng/ml under serum-free conditions for 10 minutes at 37°C. The cells were rinsed with ice-cold PBS and collected with lysis buffer containing protease inhibitors. The samples were analyzed by Western blot using anti-phosphorylated ERK, anti-total ERK, and mouse monoclonal anti-Hn1 antibodies.

**Treatment of B16.F10 cells with MAPK Pathway Inhibitors**

B16.F10 cells were plated and transduced twice with Hn1- or scrambled-siRNA (M.O.I. 5000) as described above. Three hours after viral transduction, on the second day of viral transduction, cells were supplemented with DMEM containing 20% FBS and either the p38 MAPK inhibitor SB203580 (20mM), the MEK inhibitor U0126 (10mM), or DMSO. Protein lysate was collected 48 h later with lysis buffer containing protease inhibitors for Western blot analysis as described above.

**Treatment of B16.F10 Cells With Forskolin and α-Melanocyte-Stimulating Hormone**

B16.F10 cells were plated and transduced twice with Hn1- or scrambled-siRNA (M.O.I. 5000) as described above. Three days after the first transduction, cells were rinsed with serum-free DMEM and treated with 1 μM forskolin or α-Melanocyte-Stimulating Hormone (α-MSH), or with an equivalent volume of DMSO. Cells were placed back in the incubator for 8 h and collected with lysis buffer containing protease inhibitors for Western blot analysis as described above.
Treatment of RAW264.7 Cells With RANKL

RAW264.7 cells (100 µl at a density of 100 cells/µl) were pipetted onto the center of each well on a 24-well dish. After 10 minutes, the medium was replaced with DMEM containing 10% FBS and 50ng/ml of RANKL. After five days in a 37°C incubator containing 5% CO₂, cells were viewed under the microscope for signs of multinucleation indicating osteoclast differentiation. When significantly multinucleated (5-7 days after plating), protein lysates were collected for Western blot analyses or cells were fixed for immunocytochemical analysis as described above.

Transfection of HEK293 Cells

HEK293 cells were plated at two different densities (150,000 and 250,000 cells per well) on a 12-well dish. The medium was changed on the next day. The following day, cells were transfected using Lipofectamine 2000 and Opti-MEM I (Invitrogen, San Francisco, CA). The DNA used to transfect the cells included the human HN1, murine Hn1 and empty pCDNA vector. For each well, 1 µg of each DNA was mixed with Opti-MEM I for a total volume of 50ul. Simultaneously, Lipofectamine 2000 (5 µl) was mixed with 45 µl Opti-MEM I. The two mixes were combined and incubated at room temperature for 45 minutes in the hood. Cells were rinsed with serum-free DMEM and 400 µl of serum-free DMEM was added to each well. The 100 µl from each DNA/Lipofectamine2000/Opti-MEM I mix was added to each well. Five hours later, the cells were supplemented with 500 µl DMEM containing 20% FBS. The medium was replaced with DMEM containing 10% serum the following day and protein samples were collected on the following two days (higher cell density one day after, and lower cell density the next day) for Western blot analysis as described above.
Statistical Analysis

For analysis of statistical significance, the data were subjected to student’s t-test (two-tailed distribution and two-sample equal variance test). Most experiments were conducted at least three times.
Figure 2-1. Transduction efficiency of different AAV serotypes on GL261 cells. Representative fluorescence microscopy images of GL261 cells transduced with GFP-expressing recombinant self-complimentary (sc) AAVs pseudotyped with capsid proteins from serotypes 1, 2, 3, 5, 6, and 8. Images of GL261 cells transduced with GFP-expressing scAAV4, 7 and 9 are not shown.
CHAPTER 2
HN1 EXPRESSION IN THE MURINE GL261 GLIOMA MODEL

Introduction

The rapid growth and invasive characteristics of gliomas can be caused by genetic and external factors that alter intracellular mechanisms responsible for controlling cell proliferation and repair. Identifying the roles of the various genes involved in the aberrant growth properties of tumors is a valuable goal toward uncovering mechanisms of tumorigenesis. The little-studied hematopoietic- and neurologic-expressed sequence 1 (Hn1) gene encodes a small protein that is expressed throughout several biological models of growth. When comparing the expression of genes involved in regenerating and non-regenerating models of injured motoneurons, our lab identified the marked upregulation of Hn1 mRNA and protein in axotomized adult facial motor and vagus nerves, both of which regenerate after injury (Zujovic et al. 2005). The duration of the increased expression correlated with the time required for the neurons to regenerate completely. Moreover, an ortholog of Hn1 in the Japanese common newt is upregulated during dedifferentiation of their regenerating retinal pigment epithelial cells (Goto et al. 2006).

Numerous tissues, including the brain, spinal cord, heart, thymus, spleen and testis, manifest high levels of Hn1 mRNA during murine embryonic development (Zujovic et al. 2005). Although the expression of Hn1 throughout the brain of the newborn rat and mouse is diffuse and intense, in adulthood it becomes restricted to the hippocampus, cortex, and cerebellum, regions of the brain that exhibit high plasticity (Zujovic et al. 2005). Furthermore, HN1 is one of four genes that distinguish epithelial ovarian carcinoma cells from normal ovarian surface epithelial cells (Lu et al. 2004). While the specific function of Hn1 is not known, this collective information suggests that this gene is involved in processes associated with cell proliferation, repair and/or growth.
The murine Hn1 cDNA was first isolated from embryonic erythroid cells derived from yolk sac blood islands. The naming of this gene followed the realization that it is highly expressed in hematopoietic cells and fetal brain (Tang et al. 1997). Hn1 has a unique protein sequence consisting of 154 amino acids and it is conserved among numerous species including humans, rodents, primates, cattle, birds, fish, amphibians, and insects. Murine Hn1 is found on chromosome 11, which is often considered parallel to human chromosome 17, where the human ortholog is located (Tang et al. 1997; Zhou et al. 2004).

Here, we investigated the expression of Hn1 in the murine GL261 glioma model, which was established in C57BL/6 mice after an intracranial injection of 3-methylcholanthrene (Zimmerman and Arnold 1941a). The tumor was originally maintained in vivo by serial transplantation of small tumor pieces onto syngeneic C57BL/6 mice and the GL261 cell line was established thereafter (Ausman et al. 1970; Szatmari et al. 2006). GL261 cells exhibit rapid in vitro growth rates, they lack contact inhibition and develop an aggressive tumor when injected into their syngeneic host (San-Galli et al. 1989; Weiner et al. 1999). The murine GL261 glioma model is appropriate for the study of treatments against glioma because it shares numerous characteristics with human gliomas (Szatmari et al. 2006; Sidransky et al. 1992; Ishii et al. 1999; Bos 1989). They are invasive but do not metastasize, have a high tumor take rate, mutated p53 and K-ras, and upregulated c-myc and p53 (Szatmari et al. 2006). The results reported here establish that Hn1 protein is expressed in the murine GL261 glioma model. In addition, an adeno-associated virus (AAV) engineered to express a recombinant silencing RNA (siRNA) sequence that targets and degrades murine Hn1 mRNA in GL261 cells was developed. With this tool, the effect of Hn1 depletion on the in vitro and in vivo growth of GL261 cells and tumors was evaluated.
Results

Hn1 mRNA and Protein are Expressed in GL261 Cells and Tumors

Since Hn1 expression has been reported in biological models involving cellular growth and proliferation, the expression of this gene was explored in GL261 cancer cells. Northern blot analyses identified two mRNA species of 0.7 and 1.4 kb (Figure 3-1A). Hn1 protein in GL261 cells cultured in vitro was detected by Western blot analysis using a rabbit polyclonal anti-murine Hn1 antibody. This antibody identified a 25 kDa band in these cells that co-migrated on SDS-PAGE with a recombinant form of Hn1 (Figure 3-1B) expressed in HEK293 cells, which do not normally express Hn1 protein. To further validate the specificity of the Hn1 antibody, GL261 cells were treated with a serotype 6 adeno-associated virus (AAV6) expressing a silencing RNA (siRNA) sequence against murine Hn1 mRNA. Western blot analysis of these samples collected 2 days later revealed that the 25 kDa protein recognized by the Hn1 antibody was no longer present in the samples treated with the Hn1-siRNA AAV6 (Figure 3-1C).

The in vitro findings were complemented by in situ hybridization (ISH) and immunohistochemical analyses of GL261 tumors established in murine (C57BL/6) brains (Figure 3-2). Strong hybridization signals were evident within the GL261 intracranial tumor mass, indicating high levels of Hn1 mRNA (Figure 3-2A-D). Hn1 protein expression in GL261 tumors was detected by immunohistochemical methods using the Hn1 antibody (Figure 3-2E). The tumor was generated in a C57BL/6 mouse that expresses green fluorescent protein (GFP) in all the CX3CR1-expressing cells. In this model, the GFP/CX3CR1-expressing cells represent glioma-infiltrated microglia (Liu et al. 2008). Hn1 protein did not co-localize with the GFP/CX3CR1-expressing microglia, consistent with its expression within the tumor cells.
Impact of Hn1 Depletion on the Growth of GL261 Cells and Tumors

To evaluate the \textit{in vitro} proliferation rates of cells with and without Hn1 protein, GL261 cells were treated independently with the Hn1-siRNA AAV6, control H1 AAV6 (each at an M.O.I. of 5000), or no virus. Cell counts were obtained at 2, 3, 4, 6, and 8 days after the addition of virus. Protein lysates from each sample were also collected at each time point for Western blot analysis in order to confirm the effect of the siRNA on Hn1 protein expression (data not shown). The \textit{in vitro} proliferation rate of the Hn1-depleted GL261 cells did not differ from either the control-treated or the untreated cells (Figure 3-3). A parallel experiment was conducted \textit{in vivo} on C57BL/6 mice. Twenty mice were implanted intracranially with GL261 cells pretreated with either Hn1-siRNA AAV6 or the control H1 AAV6. The brain tumors were allowed to develop for 20 days, at which time all the mice were euthanized. Excised brains were cryosectioned and their tumor volumes were calculated based on the sum of the areas of each tumor section (Figure 3-4A). Representative sections of the two tumor groups were stained with hematoxylin and eosin (H&E stained) (Figure 3-4B). Tumor volumes were significantly smaller (p<0.05) in the animals implanted with Hn1-depleted cells. Hn1 mRNA expression as measured by \textit{in situ} hybridization was variable in tumors with and without Hn1 (data not shown).

Discussion

The main findings reported here include the identification of Hn1 mRNA and protein expression in GL261 cells cultured \textit{in vitro} as well as in murine intracranial tumors established from these cells. While the \textit{in vitro} proliferation of cells lacking Hn1 was not altered, tumors derived from Hn1-depleted GL261 cells were significantly smaller than tumors generated from Hn1-expressing cells. Collectively, the data identify Hn1 as a novel protein involved in the biology of malignant gliomas.
Gliomas are very difficult to treat because of the delicate intracerebral environment in which they develop and their highly invasive and immunosuppressive properties. Identifying genes involved in the regulation of tumor growth, as well as the invasive and immunosuppressive properties of gliomas is a valuable goal toward developing novel anti-glioma therapies. Previously published results indicate that Hn1 protein is expressed in numerous tissues during development, in highly regenerating nervous tissue, and in at least one type of cancer, the human ovarian carcinoma (Zujovic et al. 2005; Goto et al. 2006; Lu et al. 2004). These varied Hn1-expressing scenarios are suggestive of a role for Hn1 in mechanisms involved in cell proliferation or in maintaining cells in a dedifferentiated state common to cell repair and development. As such, we explored the expression of Hn1 in a murine model of malignant glioma and determined that this type of brain tumor expresses the Hn1 protein. The murine GL261 model of malignant glioma reproduces many aspects of the high-grade human glioblastoma multiforme including the latter’s rapid growth, invasive characteristics, and genetic alterations (Szatmari et al. 2006; Sidransky et al. 1992; Ishii et al. 1999; Bos 1989). Identifying HN1 expression in human gliomas, as will be shown in a later chapter, adds to the existing evidence that this is an appropriate murine model to study human gliomas. Uncovering the cellular function of HN1 in human malignant gliomas will be facilitated by parallel studies in this murine model.

Insight into the function of Hn1 in gliomas was obtained from experiments utilizing a recombinant AAV engineered to express an anti-Hn1 siRNA. The Hn1-siRNA efficiently inhibited the expression of Hn1 protein in the GL261 cells within two days after addition of the virus, suggesting a relatively short half-life of the protein, possibly less than 24 hours. Despite a lack of differences in the in vitro proliferation rates of GL261 cells with and without Hn1, Hn1-
depleted GL261 cells formed intracranial tumors that were significantly smaller than those established from control-treated cells. As a group, the tumors from Hn1-depleted cells developed tumors with smaller intracranial volumes. In addition, a greater number of animals had no visible tumors after implantation with Hn1-depleted cells. The difference between the in vitro and in vivo growth effects of Hn1 depletion suggests that Hn1 does not play a direct role in regulating the proliferation rate of the GL261 cells and indicates that the function of Hn1 is dependent upon the environment in which the tumor cells grow and invade normal brain tissue. It is noteworthy to mention that Hn1 expression on the GL261 tumors, as viewed by immunohistochemistry and in situ hybridization, was particularly higher on the edge of the tumors. This is an area where cells proliferate faster than on the center of the tumor (Pescarmona et al. 1999) and where the interaction with factors from the surrounding environment, e.g. membrane-type 1 matrix metalloproteinase and matrix metalloproteinase 2 (MT1-MMP/MMP2), is critical for the invasive capabilities of the tumor (Seiki et al. 2003). The development of the anti-Hn1 antibody as well as the highly efficient anti-Hn1-siRNA AAV6 will facilitate future studies addressing the specific cellular function of Hn1 in murine cancer cells and other biological processes.
Figure 3-1. Hn1 is expressed in the murine GL261 glioma cell line. A) Northern blot analysis of Hn1 mRNA in GL261 cells. The migration of the 18S rRNA is indicated. B) Western blot analysis of HEK293 cells (HEK), HEK293 cells transfected with a recombinant murine Hn1 protein (HEK-Hn1), and GL261 cells using the rabbit polyclonal anti-Hn1 antibody. C) Western blot analysis, using the anti-Hn1 antibody, showing Hn1 expression in GL261 cells transduced at an M.O.I. of 5000 with either a Hn1-siRNA AAV6 (siRNA) or control H1 AAV6 (H1-control).
Figure 3-2. Hn1 is expressed in GL261 brain tumors of C57BL/6 mice. Sections were derived from tumor bearing brains 20 days post-GL261 cell implantation. Panels A and B depict low resolution ISH analysis of Hn1 mRNA in GL261 tumors using A) anti-sense and B) sense Hn1 riboprobes. Panels C and D depict higher resolution silver grain analysis of adjacent sections from a GL261 brain tumor hybridized with C) anti-sense and D) sense Hn1 riboprobes. High silver grain density on the left side of the section indicates Hn1 expression within the tumor cells. Sections were counterstained with H&E following ISH. E) Immunohistochemical analysis using a rabbit polyclonal anti-Hn1 antibody showing Hn1 (red) in the GL261 tumor cells and not the GFP+ microglia (Greene et al.). Sections were counterstained with DAPI nuclear stain (blue).
Figure 3-3. Hn1 depletion does not affect \textit{in vitro} proliferation rates of GL261 cells. \textit{In vitro} growth rates of GL261 cells transduced with either Hn1-siRNA AAV6 (grey), control H1 AAV6 (Hsu et al.) at an M.O.I. of 5000, or no virus (white). There was no difference in the \textit{in vitro} growth rates of GL261 cells with or without Hn1.
Figure 3-4. Hn1 depletion affects growth of GL261 tumors *in vivo*. **A**) Comparison of the growth of *in vivo* GL261 tumors established from cells pretreated with either control or Hn1-siRNA-expressing AAV6. The scatter-plot shows individual tumor volumes as well as means and SEMs. The difference between the mean volumes of tumors derived from Hn1-expressing and Hn1-depleted cells was statistically significant (p<0.05). **B**) H&E stained representative panels of tumors established from Hn1-expressing (control) and Hn1-depleted (siRNA) GL261 cells.
CHAPTER 4
MECHANISM OF HN1 IN THE MURINE B16.F10 MELANOMA MODEL

Introduction

The regulation of proliferation and differentiation of cells of melanocytic origin is a complex phenomenon. The rapidly invasive and metastatic nature of melanomas distinguishes them from their precursors, the skin melanocytes. Differentiated melanocytes contain lysosome-like organelles called melanosomes, which store the melanogenic enzymes tyrosinase, Trp1 and Trp2 (or DCT) that are necessary for the synthesis of the pigment melanin (Lin and Fisher, 2007). Melanosomes are transported by microtubules from the perinuclear region of the cell toward the periphery where they bind Rab27a, a small GTPase that is necessary for their association with actin filaments in order to secrete melanin onto the surrounding keratinocytes of the epidermis (Hume et al., 2001). These melanogenic and secretory properties coupled with cell cycle arrest are characteristic features of differentiated melanocytes. Melanocyte differentiation can be induced by phosphorylated p38 MAPK and prevented by ERK phosphorylation (Englaro et al., 1998; Saha et al., 2006; Wu et al., 2000). Understanding the various factors that distinguish melanoma tumorigenesis from melanocyte differentiation is a valuable goal in developing novel therapies against melanomas.

The hematopoietic-and neurologic-expressed sequence 1 (Hn1) gene encodes a small protein of unique amino acid sequence whose function has not yet been elucidated (Tang et al., 1997). The only other mammalian-expressed gene that shares high sequence similarity with HN1 is called HN1-like (HNIL) and its function is also unknown (Zhou et al., 2004). In rodents, Hn1 is widely expressed in numerous tissues during embryonic development. In the adult brain, Hn1 expression is restricted to regions of high plasticity, including the hippocampus, cortex and cerebellum. Hn1 is upregulated in nervous tissues that regenerate, including axotomized adult
rodent facial motor and vagal nerves (Zujovic et al., 2005). In addition, an ortholog of Hn1 in the Japanese newt is induced in dedifferentiating retinal pigment epithelial cells that arise subsequent to surgical removal of the neural retina (Goto et al., 2006). Hn1 has also been reported as one of four genes that distinguish human ovarian carcinoma from healthy ovarian epithelial tissue (Lu et al., 2004). As described in the previous chapter, Hn1 has also been found to increase the in vivo growth of murine GL261 tumors, since intracranial implantation of Hn1-depleted murine GL261 glioma cells into mice results in tumor volumes that are significantly smaller than those established from Hn1-expressing cells (Laughlin et al. 2009). The reported expression of Hn1 in models of development, regeneration, plasticity and cancer suggests that this gene is critical for maintaining a stage in cellular development and growth that precedes differentiation. The high conservation of this gene across multiple species suggests that its function may be essential for survival.

This study was undertaken to evaluate Hn1 expression and function in a murine model of melanoma. The expression of Hn1 mRNA and protein was identified in the murine B16.F10 melanoma cell line and in tumors formed from these cells. The original B16 cell line was derived from a spontaneous melanoma on the ear of a C57BL/6 mouse (Stephenson and Stephenson, 1970). The B16.F10 cells were established from this cell line and selected for their high metastatic potential (Cranmer et al., 2005; Fidler, 1973). The cells exhibit a high rate of proliferation but express proteins that are phenotypically associated with differentiated melanocytes. Using a highly efficient anti-Hn1 silencing RNA (Hn1-siRNA) to inhibit the expression of Hn1 in these cells, it was determined that this gene plays a role in the regulation of melanogenesis and cell growth.
Results

Hn1 mRNA and Protein are Expressed in B16.F10 Murine Melanoma Cells and Tumors

Northern blot analysis of extracts from B16.F10 cells identified two Hn1 mRNA species of 0.7 and 1.4 kb (Figure 4-1A), both of which are large enough to encode the 154 amino acid protein. Hn1 protein in B16.F10 cells cultured in vitro was also detected by Western blot analysis using either mouse monoclonal or rabbit polyclonal anti-murine Hn1 antibodies. Both antibodies used for Western blot analysis identified a single protein band of equal molecular weight in the B16.F10 cells (Figures 4-1B and 4-2A). The apparent molecular mass of the protein was 25 kDa, which is about 9 kDa larger than the calculated molecular mass based on the primary amino acid sequence. The in vitro findings were complemented by in situ hybridization (ISH) and immunohistochemical analyses of B16.F10 tumors established in murine C57BL/6 dermal tissue (Figure 4-1C-H). Strong hybridization signals were evident within the tumor mass, indicating high levels of Hn1 mRNA (Figure 4-1C). Hybridization with sense riboprobes established the specificity of the anti-sense riboprobe (Figure 4-1D). These skin melanomas, visualized by light microscopy (Figure 4-1E) also expressed Hn1 protein as detected by immunohistochemical analysis using the rabbit polyclonal anti-Hn1 antibody (Figure 4-1F).

Visualization of Cellular Localization of Hn1 and Inhibition of its Expression by Hn1-siRNA

To study the effects of depleting Hn1 in B16.F10 cells, a recombinant AAV serotype 6 (AAV6) was engineered to express an anti-murine Hn1 siRNA. To test the efficiency of this siRNA, B16.F10 cells were transduced with AAV6 containing either an anti-Hn1 siRNA or a non-specific scrambled-siRNA at a M.O.I. of 5000. Cell lysates collected two days after viral treatment were subjected to Western blot analysis using the rabbit polyclonal anti-Hn1 antibody (Figure 4-2A). The Hn1-siRNA very efficiently inhibited the expression of Hn1 protein in the
B16.F10 cells. Lysates from scrambled-siRNA-treated and untreated cells had similar levels of Hn1 protein. Immunohistochemical analysis of cultured B16.F10 cells determined that Hn1 protein resides throughout the cell, with a prominent nuclear localization (Figure 4-2B); the anti-Hn1 immunoreactivity was diminished in cells treated with the Hn1-siRNA.

**Effect of Hn1 Depletion on Melanogenesis and Melanin Secretion**

Hn1-depleted B16.F10 cells synthesized and secreted more melanin than Hn1-expressing cells. This phenomenon was not observed after using a M.O.I. of the control virus ten times greater than what was used for the Hn1-siRNA-AAV6. The qualitative increase in melanin secretion (Figure 4-3A) was confirmed by quantification of melanin levels in the medium. A significant increase in the levels of melanin in the medium from Hn1-depleted cells was evident by three days after viral treatment (Figure 4-3B). The increase in melanin was also visible by light microscopy within the cells cultured *in vitro*, suggesting that Hn1-depletion not only affected the secretion of melanin, but also its synthesis (Figure 4-3C).

The expression levels of several melanogenic proteins, including tyrosinase and the related proteins, Trp1 and Trp2, were analyzed in cells treated with Hn1- or scrambled-siRNAs to determine if changes in these proteins could account for the increased production of melanin by the Hn1-depleted cells. Immunohistochemical analyses using rabbit polyclonal antibodies specific to tyrosinase, Trp1 and Trp2 indicated that tyrosinase and Trp2 expression were upregulated in cells lacking Hn1; the level of Trp1 was unchanged (Figure 4-4A). These observations were confirmed by Western blot analyses using the same antibodies (Figure 4-4B). The upregulation of tyrosinase was also seen when Hn1 was depleted with the Hn1-siRNA-B (data not shown).

The effect of Hn1 depletion on a process associated with melanosomal transport and secretion was also evaluated. Rab27a, a small GTPase that forms a complex with myosin Va and
melanophilin, is essential for melanin secretion by enabling the association of melanosomes with actin filaments (Hume et al., 2007; Hume et al., 2001). The expression and localization of Rab27a was analyzed in cells with and without Hn1. While there was no difference in total levels of Rab27a, the association of Rab27a with actin was increased in the absence of Hn1 (Figure 4-4C). Immunohistochemical analysis showed enhanced co-localization of actin and Rab27a in the Hn1-depleted cells (Figure 4-4D).

**Hn1 Depletion Induces G1/S Cell Cycle Arrest**

The *in vitro* proliferation rates of cells with and without Hn1 were compared. Hn1-depleted cells grew significantly slower than cells treated with either the scrambled-siRNA or no virus (Figure 4-5A); the difference was visible by three days after viral treatment. A cell cycle analysis of cells two and three days after siRNA treatment, revealed significant G1/S arrest in the Hn1-depleted cells (Figure 4-5B). Compared to Hn1-expressing cells, a higher fraction of the Hn1-depleted cells were in G1 phase and a corresponding lower percentage of cells were in S phase. A variety of cell cycle-regulating proteins were then evaluated by Western blot analyses (Figure 4-5C). While the total level of retinoblastoma protein (Rb) did not change, lower levels of phosphorylated forms of Rb were present in cells lacking Hn1, in particular phospho-Ser795. Consistent with the decreased reactivity of the anti-phospho-Rb antibodies, the anti-total Rb antibody detected two forms of Rb in Hn1-depleted cells, with the lower band representing less phosphorylated forms of the protein. The level of cyclin D1 was increased in Hn1-depleted cells while both cyclin A and cyclin E were unaltered by the lack of Hn1. The expression of p21 was elevated, whereas p27 levels were reduced in the absence of Hn1.

The expression and phosphorylation states of various cell growth signaling molecules including c-Met, Akt, and the MAPKs ERK (p42/44) and p38 were also evaluated in B16.F10 cells with and without Hn1 using Western blot analyses (Figure 4-6). Expression of c-Met was
reduced in cells lacking Hn1 while total and phosphorylated Akt levels were unchanged (Figure 4-6A). Despite the c-Met reduction, the ability of its ligand, hepatocyte growth factor (HGF), to stimulate ERK phosphorylation was not altered (Figure 4-6B). However, reduced basal levels of ERK phosphorylation were observed, while levels of phosphorylated p38 were highly increased in the Hn1-depleted cells (Figure 4-6C). The increased p38 phosphorylation was also seen when Hn1 was depleted with Hn1-siRNA-B (data not shown), and it was independent of serum content in the culture media (data not shown). Moreover, conditioned media from Hn1-depleted cells had no effect on the basal levels of phosphorylated p38 in naïve B16.F10 cells (data not shown).

**Effect of Hn1 on Transcription Factors MITF and USF-1**

The transcription factors MITF and USF-1 have been reported to regulate the differentiation of melanogenic cells by activating the transcription of melanogenic enzymes and arresting the cell cycle. MITF can be activated by either p38 phosphorylation or ERK hypophosphorylation, whereas USF-1 can be activated by phosphorylation of p38 or ERK. Because of the collective changes seen in cell cycle arrest, p38 and ERK phosphorylation, p21, p27 and melanogenic protein expression, it was hypothesized that MITF, and possibly USF-1, would also be upregulated in Hn1-depleted cells. Western blot analyses of cells with and without Hn1 were conducted with antibodies against MITF and USF-1 (Figure 4-7). Unexpectedly, the results indicated that in the absence of Hn1, MITF was downregulated completely while USF-1 expression and phosphorylation decreased slightly.

**Effect of p38 MAPK and MEK Inhibitors on MITF, USF-1 and Tyrosinase**

To test whether MITF expression could be rescued by blocking ERK or p38 MAPK phosphorylation, B16.F10 cells with and without Hn1 were treated with SB203580 and U0126, inhibitors of p38 and MEK respectively. The expression of USF-1 and tyrosinase were also evaluated (Figure 4-8). In the scrambled-siRNA-treated cells, MEK inhibition increased levels of
MITF and decreased USF-1 phosphorylation. Inhibition of p38 had no effect on these transcription factors in cells expressing Hn1. In cells lacking Hn1, MEK inhibition by U0126 also increased MITF levels, significantly rescuing the decreased expression of this transcription factor as a result of Hn1 depletion. USF-1 phosphorylation was decreased in Hn1-depleted cells. Inhibition of p38 by SB203580 also partially rescued MITF expression and had no effect on USF-1. Tyrosinase expression was elevated by MEK inhibition and unchanged by p38 inhibition. Hn1 levels were not affected by any of the inhibitors.

**Effect of Forskolin and α-MSH on MITF**

To test whether Hn1 depletion had disrupted the cAMP-regulation of MITF expression, cells with and without Hn1 were treated with either the melanocyte stimulating hormone, α-MSH, or with forskolin, both of which stimulate cAMP synthesis (Figure 4-9). Hn1 levels were not impacted by either forskolin or α-MSH. However, treatment with either α-MSH or forskolin increased the expression of MITF in Hn1-depleted cells. Therefore, Hn1 depletion does not affect the ability of cAMP to activate MITF expression.

**Effect of Hn1 Expression on β-catenin Expression**

Activation of the Wnt/β-catenin pathway can also induce MITF expression in melanoma cells. To test whether Hn1 was interfering with β-catenin-regulated MITF expression, the effect of Hn1 depletion on β-catenin expression was evaluated by Western blot analysis (Figure 4-10). However, there was no change in total expression of β-catenin, indicating that the downregulation of MITF caused by Hn1 depletion was not due to decreased β-catenin expression.
Discussion

Published information on Hn1 has largely been confined to studies of the expression and localization of this gene in various tissues. Here, we provide additional data that indicate that B16.F10 melanoma cells and tumors express Hn1 mRNA and protein. Moreover, the main findings reported here establish that inhibition of Hn1 expression in these cells suppresses cell proliferation and increases melanogenesis. The molecular basis for these events was also evaluated. These data, coupled with previous reports demonstrating Hn1 expression in developing tissues, regenerating tissues, plasticity, and cancer (Goto et al. 2006; Laughlin et al. 2009; Lu et al. 2004; Zujovic et al. 2005), suggest that this protein plays an important role in processes associated with transitions in cell differentiation.

The pigmented property of the B16.F10 cells was especially useful at identifying a role for Hn1 in this cancer cell line since increased melanin production was evident within the cells and the culture medium shortly after depletion of Hn1 protein. The enhanced melanogenesis in the Hn1-depleted cells was explained by the increased expression of the melanogenic enzymes tyrosinase and Trp2. The higher levels of secreted melanin detected in the medium of Hn1-depleted cells also suggested that processes associated with melanosomal transport were affected by the loss of Hn1. In order to secrete melanin onto keratinocytes, the surrounding cells of the epidermis, melanosomes are transported from the perinuclear region of the cell towards the periphery where they bind actin filaments via a Rab27a–myosin Va–melanophilin complex (Hume et al., 2007; Hume et al., 2001). The enhanced interaction of Rab27a and actin observed in the Hn1-depleted cells is therefore an additional mechanism contributing to the increased secretion of melanin. The shift to a more pronounced melanogenic phenotype in the Hn1-depleted cells suggests that Hn1 normally functions to inhibit mechanisms associated with the transition towards the more differentiated melanocyte phenotype.
Further evidence in support for a role of Hn1 in regulating the differentiation of these melanoma cells is apparent from the growth inhibition observed in B16.F10 cells lacking Hn1 protein. This is consistent with in vivo studies of murine gliomas established from Hn1-depleted GL261 cells, which form significantly smaller tumors than Hn1-expressing cells, even though GL261 cells in vitro grow at equal rates whether or not they express Hn1 protein (Laughlin et al., 2009). The difference between the in vitro growth of GL261 and B16.F10 cells with and without Hn1 could be due to environmental and immune factors that influence in vivo GL261 tumor growth, or to the slower in vitro growth rate of GL261 cells compared to B16.F10 cells. A more prolonged analysis of proliferation of the GL261 cells may be required in order to see significant differences in the in vitro growth rates of Hn1-expressing and -inhibited GL261 cells.

Depletion of Hn1 results in G1/S cell cycle arrest that is consistent with the observed reduction in phosphorylation of retinoblastoma (Rb) protein, elevated cyclin D1, increased p21 and reduced levels of p27. Phosphorylation of Rb is necessary to release the transcription factor E2F, thus permitting it to drive the expression of genes that allow progression from the G1 phase to the S phase of the cell cycle (Caygill et al. 2004; Greene et al. 2007). One of the factors that can phosphorylate Rb is a complex formed by cyclin D1 and cyclin-dependent kinases 4 and 6 (cdk4/6) (Schulze et al. 1995). For this, cyclin D1 must be highly expressed during the G1 phase of dividing cells. The detected Rb hypophosphorylation and subsequent G1 arrest of the Hn1-depleted cells was likely independent of cyclin D1 levels, since high levels of cyclin D1 were detected in these cells. Other proteins that can regulate Rb phosphorylation are p21 and p27, which can bind and inhibit cdk/cyclin-dependent phosphorylation of Rb. Increased p21 and reduced p27 levels were evident in the Hn1-depleted B16.F10 cells. The expression of p21 can be driven by the basic helix-loop-helix-leucine zipper (bHLH-LZ) microphthalmia transcription
factor (MITF), which is expressed in cells of melanocytic origin (Carreira et al. 2005). On the other hand, an inverse relationship between MITF and p27 expression has been reported in human melanocytes and melanoma tissues. MITF activates the expression of Dia1, which regulates Skp2 and consequently promotes degradation of p27 (Carreira et al. 2005; Carreira et al. 2006). High levels of MITF induce a p21-dependent G1 cell cycle arrest whereas lower MITF levels induce a p27-dependent G1 cell cycle arrest (Carreira et al. 2006). Therefore, MITF expression was expected to increase in the absence of Hn1.

The reduced levels of basal ERK phosphorylation and increased levels of phosphorylated p38 evident in Hn1-depleted cells supported this hypothesis. MITF expression is positively regulated by p38 phosphorylation (Saha et al., 2006). Conversely, ERK phosphorylation elicits transient activation of MITF followed by its proteasomal degradation (Saha et al., 2006; Wu et al., 2000). In mouse melanoma cells, the consequences of ERK activation on MITF activity are predominantly inhibitory (Wellbrock et al., 2008). ERK is activated in up to 90% of human melanomas and its sustained activity in melanocytes leads to proliferation (Wu et al., 2000). Moreover, pharmacological inhibition of the ERK pathway triggers B16.F10 cell differentiation (Englaro et al., 1998). There are many factors that can result in ERK activation, among them is stimulation of the c-Met receptor by its ligand, hepatocyte growth factor (HGF). However, HGF-stimulation of ERK phosphorylation through this receptor was unaltered, suggesting that the change in c-Met was probably not sufficient to impact an HGF-dependent growth effect.

Careful regulation of MITF by multiple factors is necessary to manage its diverse functions. The regulation of melanogenesis and the cell cycle in melanoma is a complex phenomenon, controlled in large part by MITF (Carreira et al., 2005). This transcription factor can also regulate survival, proliferation, melanocyte lineage commitment, melanogenic protein
expression, and differentiation, but it has also been reported as a marker for melanoma (Gray-Schopfer et al. 2007; King et al. 1999; Levy et al. 2006; Wu et al. 2000). The collective changes seen in p38 and ERK phosphorylation, p21, p27 and melanogenic protein expression, suggested that MITF activity would be increased in Hn1-depleted cells (Carreira et al., 2005; Carreira et al., 2006). Contrary to what was expected, Western blot analysis using an anti-MITF antibody showed that this was not the case, and that in fact, MITF levels decreased with Hn1 depletion. Stimulation of cAMP synthesis with either forskolin or α-MSH treatment rescued MITF expression, indicating that the MITF gene can still be regulated by CREB in the absence of Hn1. This was not surprising, considering that MITF is necessary for cAMP-induced melanogenesis (Bertolotto et al. 1998). Also, inhibition of p38 and ERK resulted in increased MITF expression in cells lacking Hn1. The effect of p38 inhibition on MITF may initially seem unexpected because p38 has been reported to increase expression of MITF. However, the SB203580 compound used to inhibit p38 in these experiments can also inhibit JNK, which can cause hypophosphorylation of CREB, resulting in MITF downregulation and subsequent tyrosinase reduction (Bu et al. 2008; Lisnock et al., 2000). The expression of β-catenin in cells with and without Hn1 was also evaluated, since β-catenin can also activate MITF expression via the LEF-1/TCF transcription factors (Saito et al. 2003; Widlund et al., 2002). However, there were no changes in the total expression of this protein, indicating that Hn1-depletion does not affect MITF in a β-catenin-dependent manner. Visualizing the expression of β-catenin within B16.F10 cells with and without Hn1 by immunocytochemical analyses may be informative, since its nuclear localization is necessary for activation of MITF expression in B16.F10 melanoma cells (Chien et al., 2009).
The effect of Hn1 depletion was also examined on an alternative downstream target of p38, a more constitutively expressed transcription factor of the bHLH-LZ family, the upstream stimulating factor 1 (USF-1) (Corre and Galibert, 2005; Corre et al., 2004). USF-1 is necessary for embryonic development and for the UV-induced melanogenic response, and it is involved in regulating cell cycle transitions. Loss of its transcriptional activity has been detected in other cancer models including breast cancer cell lines, demonstrating its importance in cell proliferation control (Corre and Galibert, 2005). Western blot analyses of USF-1 in B16.F10 cells with and without Hn1 indicated that USF-1 expression and phosphorylation are diminished slightly in the absence of Hn1 protein. This was not surprising, considering that ERK can activate USF-1 activity (Renault et al. 2005; Samarakoon and Higgins 2008). However, it may indicate that ERK inactivation has a greater effect on USF-1 activation than increased p38 phosphorylation. This ERK-dependent activation of USF-1 is also evident in the MEK-inhibited cells, which had a much greater effect on USF-1 phosphorylation than did the p38 inhibitor. The data suggest that the growth and melanogenic effects of Hn1 depletion are independent of MITF and USF-1.

Another transcription factor of the bHLH-LZ family, TFE3, was evaluated. TFE3 can also activate tyrosinase expression and its basal expression in B16 cells is very high. It has high sequence identity with MITF and binds the same promoter motif. In B16 cells, however, TFE3 binding to this motif is prevented by an unidentified mechanism. If TFE3 were functional, the effect of MITF overexpression on tyrosinase and Trp1 would be reduced, since their basal expression from the active TFE3 would already be significantly elevated. Also, cAMP activation by forskolin or α-MSH treatment causes its downregulation (Verastegui et al., 2000). The transcription factor of the same family, TFEC, can bind TFE3 and prevent its transcriptional activation (Zhao et al., 1993). Is it
possible that Hn1 binds and inhibits TFE3 in B16 cells? TFE3 gene and protein fusions are very commonly found in some cancers (Teixeira, 2006; Wu et al., 2005). Furthermore, TFE3 overexpression in myeloblasts causes proliferation arrest and macrophage differentiation. It strongly induces terminal differentiation in hematopoietic stem cells (Zanocco-Marani et al., 2006). TFE3 expression was evaluated in B16.F10 cells with and without Hn1 and it was found to be elevated in the Hn1-depleted cells, indicating that this is likely the mechanism by which Hn1 inhibition impacted melanoma cell differentiation. Additional studies will be required to determine if and how Hn1 can regulate cell differentiation via TFE3. Given the nuclear and cytoplasmic localization of the Hn1 protein in B16.F10 cells, mechanisms that include direct interactions with transcription factors or indirect regulation of either signaling to the nucleus, e.g., via modulating MAPK pathways, or cytoplasmic-nuclear shuttling of proteins are all possibilities worthy of further exploration.

Whether other transcription factors are being regulated by Hn1 protein via increased p38 phosphorylation and decreased ERK phosphorylation will require additional studies. One additional transcription factor of interest is Sox10. It has been demonstrated that Sox10, belonging to the HMG box family of transcription factors, is capable of activating tyrosinase expression via the core enhancer element that is located upstream of the tyrosinase promoter (Murisier et al. 2007). In fact, it is necessary for proper levels of tyrosinase expression and pigmentation of melanocytic cells (Hou et al. 2006). Without it, MITF or USF-1 alone can only produce very low levels of tyrosinase expression. This core-enhancer element can also be activated by MITF and USF-1, which compete with each other for its activation. However, Sox10 binds a different consensus site and its activation of the core enhancer is independent of either MITF or USF-1. Sox10 also activates the promoter of Trp2 and the core enhancer element of Trp1. It has been proposed that Sox10 activates MITF expression, but this linear theory has
been replaced by one involving a more interactive regulatory network (Murisier et al. 2007). Moreover, Sox10 appears to be necessary for neural crest cell formation, cellular multipotency, specification, and differentiation (Kelsh 2006). It is also necessary for the survival of migrating neural crest cells and for terminal differentiation of oligodendrocytes (Wegner and Stolt 2005). Interestingly, Sox10 expression in human gliomas is lower than in normal brain tissue (Schlierf et al. 2007). It would be interesting to determine the effect of Hn1 depletion on Sox10 expression and localization in the B16.F10 cells. Is Hn1 suppressing the expression or activity of Sox10?

In conclusion, the expression of Hn1 appears to maintain B16.F10 cells in a less differentiated state that results in a more tumorigenic phenotype. The expression of Hn1 during development, neuronal plasticity and regeneration, and cancer collectively points to a role for this gene in processes involved in either undifferentiated or dedifferentiated cellular states. During development, cells divide and migrate to their specific locations prior to differentiating. The presence of Hn1 in brain regions involved in learning and memory, such as hippocampal and cortical neurons, suggests a role for this gene in the phenomenon of plasticity. Indeed, in the injury responses that lead to motoneuron and retinal regeneration, neurons and retinal pigment epithelial (RPE) cells must halt specific functions associated with a differentiated phenotype in order to undergo repair and regenerate. These periods of cellular dedifferentiation are also marked by significant increases in the expression of Hn1 (Zujovic et al. 2005; Goto et al., 2006). Interestingly, p38 hyperphosphorylation has been detected in axotomized retinal neurons of mammals, which undergo apoptosis. Blocking p38 phosphorylation with SB203580 increases the survival of axotomized neurons (Kikuchi et al. 2000). If Hn1 is to be a possible target for therapies against cancer or neurodegenerative diseases, it will be necessary to determine its precise mechanism of action, including its role upstream of the ERK and p38 MAPK pathways.
On one hand, inhibition of Hn1 may promote successful suppression of cancer growth, while alternatively, in situations requiring tissue regeneration, it may be ideal to enhance its activity.
Figure 4-1. Hn1 is expressed in B16.F10 murine melanoma cells and tumors. Northern (A) and Western blot (B) analyses of B16.F10 cell extracts showing Hn1 mRNA and protein expression. The mouse monoclonal anti-Hn1 antibody was used for Western blot analysis. The position of the 18S ribosomal RNA and calculated molecular mass of Hn1 protein (25 kDa) are shown. (C, D) In situ hybridization analysis of Hn1 mRNA within a B16.F10 skin tumor section. (C) A section was hybridized with an Hn1 anti-sense riboprobe; (D) an adjacent section was hybridized with a sense riboprobe. Scale bar: 2 mm. (E) Light microscopy of a murine B16.F10 skin tumor, tumor tissue (left) and surrounding normal tissue (right). (F) Hn1-expressing cells determined by anti-Hn1 immunohistochemistry using the rabbit polyclonal anti-Hn1 antibody. (G) DAPI-stained cell nuclei. (H) A merged image of (F) and (G). Scale bar: 50 μm.
Figure 4-2. Depletion of Hn1 protein in B16.F10 cells by an anti-Hn1 siRNA. (A) Western blot analysis of B16.F10 cells treated with scrambled-siRNA and Hn1-siRNA adeno-associated viruses at an M.O.I. of 5000 using the rabbit anti-Hn1 polyclonal antibody. Untreated cells are also shown. (B) Confocal immunofluorescence microscopy (CIM) of Hn1 protein in cells with and without Hn1. The rabbit polyclonal anti-Hn1 antibody was used for immunocytochemical analyses and DAPI was used as the nuclear stain. Scale bar: 16 µm.
Figure 4-3. Hn1 depletion in B16.F10 cells increases melanin secretion. (A) Qualitative analysis of melanin secreted by B16.F10 cells treated with Hn1-siRNA virus at and M.O.I. of 3000 or control virus at M.O.I.s of 3000 or 30,000. The higher M.O.I. of the control virus did not impact melanin secretion. (B) Quantitative analyses of melanin secretion by B16.F10 cells treated with Hn1-siRNA (triangles), scrambled-siRNA (squares), or no virus (circles). Shown are the means of triplicate samples ± SEM (*p<0.01; **p<0.00001) of a representative experiment conducted three independent times. (C) Light microscopy of Hn1-expressing (scrambled) and -depleted (siRNA) B16.F10 cells. Scale bar: 16 µm.
Figure 4-4. Hn1-depleted B16.F10 cells have increased levels of Tyrosinase and Trp2 and an enhanced association of Rab27a and actin. (A) CIM and (B) Western blot analyses of B16.F10 cells treated with the scrambled or Hn1-siRNA showing Tyrosinase (Tyr), Trp1 and Trp2 protein localization and levels. An anti-total Akt antibody was used as the loading control for the Western blot. (C) Upper panels depict Western blot analyses of total cell extracts (cell lysate) of B16.F10 cells with and without Hn1 using antibodies against Rab27a, Hn1 and total Akt, the loading control. Lower panels show the final pellets from an immunoprecipitation reaction of Hn1-expressing and -depleted B16.F10 cell lysates using an anti-actin polyclonal antibody and analyzed by Western blot analysis using the monoclonal anti-actin and anti-Rab27a antibodies. (D) CIM of Rab27a expression in B16.F10 cells with and without Hn1; Rab27a (red), actin (green), DAPI-stained nuclei (blue); arrows indicate colocalization of Rab27a and actin. Scale bars: 16 µm.
Figure 4-5. Hn1 depletion induces B16.F10 cell cycle arrest. (A) Growth rates of B16.F10 cells treated with Hn1-siRNA (triangles), scrambled-siRNA (squares), or no virus (circles). Shown are the means ± SEM (*p<0.001) of triplicate samples from a representative experiment conducted six independent times. (B) Cell cycle analysis of Hn1-expressing and Hn1-depleted cells 2 and 3 days after siRNA treatment. Two-day scrambled (black), two-day siRNA (white), three-day scrambled (horizontal line pattern), three-day siRNA (vertical line pattern). *p<0.05; **p<0.001. (C) Western blot analyses of extracts from Hn1-expressing and Hn1-depleted B16.F10 cells using antibodies specific to a variety of cell cycle proteins. An anti-total Akt antibody was used as the loading control.
Figure 4-6. Effect of Hn1 depletion on growth signaling molecules. (A) Western blot analyses of B16.F10 cells with and without Hn1 using anti-c-Met, -phosphorylated Akt (P-Akt), -total Akt and -Hn1 antibodies. (B) Western blot analyses of hepatocyte growth factor (HGF) stimulation of ERK phosphorylation in B16.F10 cells with and without Hn1. (C) Western blot analyses of B16.F10 cells treated with scrambled, Hn1-siRNA or no virus using anti-phosphorylated ERK (P-ERK), -total ERK, -phosphorylated p38 (P-p38), -total p38, and -Hn1 antibodies.
Figure 4-7. Effect of Hn1 depletion on the expression of transcription factors MITF and USF-1. B16.F10 cells were treated with the scrambled- or Hn1-siRNAs and analyzed by Western blot using antibodies against MITF and USF-1.
Figure 4-8. Effect of p38 and MEK inhibition on MITF and USF-1 in cells with and without Hn1. Western blot analyses showing the changing expression of MITF protein in cells treated with the scrambled- or Hn1-siRNA in combination with treatment with p38 or MEK inhibitors SB203580 and U0126 respectively.
Figure 4-9. Effect of cAMP-elevating agents on MITF expression in B16.F10 cells with and without Hn1. Western blot analyses show the changing expression of MITF protein in cells treated with the scrambled-siRNA, Hn1-siRNA or no virus in combination with treatment with α-MSH or Forskolin.
Figure 4-10. Effect of Hn1 depletion on β-catenin expression in B16.F10 cells. Western blot analysis of cells treated with scrambled- or Hn1-siRNA using an antibody specific for β-catenin. An antibody against total Akt was used as the loading control.
Figure 4-11. Effect of Hn1 depletion on the expression of transcription factor TFE3 in B16.F10 cells. Western blot analysis of cells treated with scrambled- or Hn1-siRNA using an antibody specific for TFE3. An antibody against actin was used as the loading control.
CHAPTER 5  
HN1 IN HUMAN TUMORS

Introduction

As demonstrated in the two previous chapters, Hn1 plays an important role in the regulation of tumor growth and cell dedifferentiation. In order to validate the significance of the extensive studies conducted in the GL261 and B16.F10 animal models, the expression of HN1 protein was evaluated in numerous types of human cancer cell lines and tissues. Western blot analyses were conducted to determine if HN1 protein is expressed in different human cancer cell lines including glioma (U87-MG and U118-MG), melanoma (SK-Mel-28, A375 and C8161), esophageal cancer (TE-1, -2, -3, -4, -5 and KYSE-140, -150, -180), ovarian carcinoma (OVCAR-3) and erythroleukemia (K562). Furthermore, high-grade human brain tumor tissue sections were subjected to immunohistochemical analyses to evaluate HN1 expression. The brain tissue samples analyzed included glioblastoma multiforme, anaplastic forms of oligodendroglioma and astrocytoma, and healthy cortex. An independent study in the laboratory of Dr. Keiran S. Smalley at the Moffitt Cancer Center & Research Institute in Tampa, FL, detected HN1 expression in several human tumor tissue samples including esophageal carcinoma and melanoma. Additionally, the laboratory of Dr. Brian Law, Department of Pharmacology and Therapeutics at the University of Florida, Gainesville, FL, also found HN1 protein expression in several human breast cancer cell lines.

The tumorigenic U87-MG and U118-MG glioblastoma cell lines were derived from grade III glioblastomas from a 44-year-old female and a 50-year-old male respectively. Both tumors were highly malignant astrocytomas and mitoses occur more commonly in these cells than in cultures of normal glia. The U87-MG tumor is characterized by necrosis and large tumor cells. In culture, cells derived from this tumor are also large and very bizarre, and they have a very slow
growth rate. The U118-MG tumor is characterized by pleomorphism and fibrosis, and the cultured cells have cytoplasmic granulation (Ponten and Macintyre 1968).

The tumorigenic human melanoma SK-Mel-28 cell line was derived from a melanoma on the skin of a 51-year-old male (ATCC, The Global Bioresource Center). In a study of the immune response elicited by 35 melanoma cell lines on their corresponding patients (the patients on whom the original tumors formed), this one elicited the highest response (Carey et al. 1976). The A375 malignant melanoma cell line was derived from a skin melanoma of a 54-year-old female (ATCC, The Global Bioresource Center). Treatment of these cells with bromodeoxyuridine (BrdU) induces reversible differentiation (Ohtani et al. 1997). The C8161 human melanoma cell line was derived from an abdominal wall metastasis. It is an amelanotic and highly metastatic cell line with a doubling time of approximately 6 days (Welch et al. 1991).

The three KYSE and TE cell lines were derived from esophageal squamous cell carcinomas (SCCs) from different patients. KYSE-150, TE-2 and TE-5 cells were derived from poorly differentiated esophageal SCCs. KYSE-140 cells were derived from a moderately differentiated SCC. KYSE-180, TE-1, -3, and -4 cells originated from well-differentiated SCCs; however, TE-3 and -4 cells were derived from metastatic lesions (Nakajima et al. 2004). The doubling times are approximately 14, 17, 24, 25, and 28 hours for the TE-2, -3, -5, -1, and -4 cells respectively, and 14, 15 and 27 hours for the KYSE-150, -180 and -140 respectively (Zhao et al. 2000).

The OVCAR-3 cell line was derived from the poorly differentiated malignant ascites of a patient with progressive adenocarcinoma of the ovary after combination chemotherapy. The cell line is tumorigenic, clonogenic in agarose and has an abnormal karyotype. Moreover, it
continues to have the characteristics of the tumor from which it originated and is therefore an excellent model to study the molecular basis of human ovarian carcinoma (Hamilton et al. 1983).

The K562 cell line originated from a 53-year-old female chronic myeloid leukemia patient in terminal phase blast crisis (Klein et al. 1976). These undifferentiated blast cells are considered very early erythroid precursors. They grow in suspension culture and their average doubling time is 12 hours (Koeffler and Golde 1980). The variety provided by this collection of human cancer cells was evaluated in relation to their HN1 expression.

Results

HN1 Protein Expression in Human Cancer Cell Lines

Western blot analysis of the human glioma cell lines U118-MG and U87-MG detected three distinct molecular weight bands that migrated with apparent molecular masses of 23, 25, and 30 kDa (Figure 5-1A). The presence of these three bands in human cell lines is consistent with the identification of three variants of HN1 in humans (GenBank accession numbers: Variant 1: NP057269; Variant 2: NP001002032; Variant 3: NP001002033). HN1 expression was also detected, in varying levels, in the KYSE-140, -150 and -180, and the TE 1, 2, 3, 4 and 5 esophageal cancer cell lines as well as the OVCAR-3 ovarian carcinoma and K562 erythroleukemia cell lines (Figure 5-1B). However, HN1 protein was not detected in any of the three tested human melanoma cell lines (Figure 5-1C).

HN1 Protein is Expressed in High-Grade Human Brain Tumors

The expression of HN1 protein was evaluated in human brain tumors. A human brain tumor tissue microarray, containing multiple WHO tumor grade III and IV infiltrating gliomas, was subjected to immunohistochemical analysis with the rabbit polyclonal anti-Hn1 antibody. The antibody detected strong HN1 immunoreactivity in human malignant gliomas including glioblastoma and anaplastic forms of astrocytoma and oligodendroglioma (Figure 5-2). HN1
expression in a cortical region of human brain was negative, except on two of the tissue samples where weak immunoreactivity was detected in neuronal cells (Table 5-1). Of the WHO grade III and IV gliomas, 75% expressed strong anti-HN1 immunoreactivity while 11% had moderate HN1 expression.

**Discussion**

Consistent with the identification of Hn1 expression in the murine GL261 glioma cell line, HN1 was detected in the U118MG and U87MG human glioblastoma cell lines as well as human high-grade glioma tissue, including glioblastoma multiforme and anaplastic forms of astrocytoma and oligodendroglioma. The localization of HN1 protein paralleled what was seen in the mouse GL261 tumors, where the tumor cells were the prominent HN1-expressing cell type. Additionally, HN1 protein was detected in multiple human cancer cell lines including eight esophageal cancer cell lines, one ovarian carcinoma and one erythroleukemia cell line. Personal communication from Dr. Keiran S. Smalley at the Moffitt Cancer Center & Research Institute indicates that HN1 expression was found independently in several human esophageal cancer and melanoma tissue samples. However, the three human melanoma cell lines analyzed here did not express HN1 protein. This was inconsistent with the high Hn1 expression evident in the murine B16.F10 melanoma cell line and may indicate differences in the tumorigenicity, proliferation, metastatic ability, or stage of differentiation in the different cells. Further investigation of the nature and phenotype of each melanoma may provide answers to why HN1 expression differs in these cells. For instance, there may be a correlation between HN1 expression and their migratory and invasive characteristics.

Hn1 expression was detected at very low levels in the KYSE-150 cells and was higher in the KYSE-140 and -180 cells. In comparison with the other two cell lines, KYSE-150 cells were derived from a less differentiated tumor that had undergone irradiation treatment. There was no
correlation between Hn1 expression and cell division times in these cells. In order of increasing intensity, the expression of Hn1 in TE cells was TE-4, -1, -5, -2, and -3. A positive correlation between proliferative activity and percentage of TE cells in S phase has been previously reported. From lower to higher percentage of cells in S phase, the order of the cells was TE-4, -1, -2, -3, and -5 (Nakajima et al. 2004). It appears that, in these cells, Hn1 expression is positively correlated to the percentage of cells in S phase, or with proliferative activity, and this is consistent with the B16.F10 data presented in chapter 4 (Figure 4-5). Also, the more recently reported doubling times for all these cell lines have a perfect negative correlation with the expression of Hn1 in these cells; in order from more slowly to more rapidly dividing cells, TE-4, -1, -5, -3 and -2 (Zhao et al. 2000). Moreover, the expression of p27 and cyclin D1 in these cells seemed to be positively correlated to Hn1 expression as well, with TE-2 and -3 being the only ones that expressed these two proteins (Nakajima et al. 2004). This, however, is contradictory to our findings, which indicated an increase in p27 and cyclin D1 as a result of Hn1 depletion in the B16.F10 cells.

Hn1 expression was high in both OVCAR-3 and K562 cells, both of which are very poorly differentiated. Whether the chemoresistant properties of OVCAR-3 cells are linked to Hn1 expression remains to be explored. Also, the high levels of Hn1 and USF-1 in the K562 cells are supportive of the data presented in chapter 4 (Giannola et al. 2000). Another possible experiment to try would be to deplete Hn1 from these cells and observe whether USF-1 phosphorylation levels decrease as they do in the B16.F10 cells. This may cause HOXB4 to decrease as well, driving the cells to a more differentiated state. For this, and for the OVCAR-3 experiment, a siRNA specific to human HN1 would need to be developed.
Figure 5-1. HN1 expression in various human cancer cells. HN1 expression was evaluated by Western blot analyses in human A) glioblastoma, B) melanoma, C) esophageal squamous cell carcinoma, D) erythroleukemia (K562) and ovarian carcinoma (OVCAR-3).
Figure 5-2. HN1 is expressed in multiple types of human high-grade glioma. A human brain tumor tissue microarray containing a variety of tumor samples was subjected to immunohistochemistry using the rabbit polyclonal anti-Hn1 antibody. Strong immunoreactivity (brown) was identified in human malignant gliomas including (A-C) glioblastoma multiforme, (D) anaplastic oligodendroglioma, and (E) anaplastic astrocytoma. (F) A region of the cortex from normal brain.
Table 5-1. Immunohistochemical analysis of Hn1 expression in human gliomas.

<table>
<thead>
<tr>
<th>Tissue type</th>
<th>Number of cases</th>
<th>Relative intensity of Hn1 immunoreactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-neoplastic (negative control)</td>
<td>7</td>
<td>Neuros 2/7 weak 5/7 absent</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Astrocytes 7/7 absent</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Oligos 7/7 absent</td>
</tr>
<tr>
<td>WHO Grade IV Tumor (Louis et al. 2007)</td>
<td>11</td>
<td>Glioblastoma Multiforme 6 strong 2 moderate 3 weak</td>
</tr>
<tr>
<td>WHO Grade III Tumor (Louis et al. 2007)</td>
<td>5</td>
<td>Anaplastic Astrocytoma 4 strong 1 moderate</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Anaplastic Oligoastrocytoma 1 weak</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>Anaplastic Oligodendroglioma 11 strong</td>
</tr>
<tr>
<td>All tumors</td>
<td>28</td>
<td>21 (75%) strong 3 (11%) moderate 4 (14%) weak</td>
</tr>
</tbody>
</table>

Strong: over 80% of cells have anti-Hn1 immunoreactivity. Moderate: 21% to 79% of tumor cells have anti-Hn1 immunoreactivity. Weak: less than 20% of cells have anti-Hn1 immunoreactivity.
CHAPTER 6
HN1 IN CELL DIFFERENTIATION

Introduction

The hematopoietic- and neurologic-expressed sequence1 (Hn1) is highly expressed in multiple tissues during rodent embryonic development and in plastic regions of the adult rodent brain (Zujovic et al. 2005). Its expression is also highly upregulated during the neural regenerative period after adult rodent facial nerve axotomy and vagotomy, as well as in the dedifferentiated retinal pigment epithelial cells during regeneration of the Japanese common newt retina (Goto et al. 2006; Zujovic et al. 2005). This knowledge, combined with the expression of Hn1 in various murine and human cancer cells and tumors, and with the data showing that Hn1 depletion causes B16.F10 melanoma cells to become more differentiated, suggests that Hn1 expression may be critical for maintaining a stage in cellular development and growth that precedes differentiation.

To test this hypothesis, a series of experiments have been designed to evaluate the effects of Hn1 expression on cell differentiation, and vice versa. The first model used was the RAW264.7 cell line, which is a transformed murine macrophage cell line established from a tumor induced by Abelson murine leukemia virus (ATCC, The Global Bioresource Center). RAW264.7 cells express high levels of the receptor activator of nuclear factor kappa B (RANK), which is expressed by osteoclast precursors. Osteoclasts are the multinucleated cells responsible for bone resorption. Differentiation of RAW264.7 cells into osteoclasts is induced by stimulation with the RANK ligand (RANKL), a member of the tumor necrosis factor (TNF) family (Hsu et al. 1999). Differentiation of RAW264.7 cells into osteoclasts by RANKL treatment is positively regulated by p38 MAPK and negatively regulated by the MEK/ERK pathway (Hotokezaka et al. 2002). Multiple transcription factors are involved in driving the differentiation of these cells.
Among them are MITF and PU.1. There is also evidence that TFE3 is involved in this pathway because mice with a null allele of TFE3 have severe osteoclast abnormalities (Sharma et al. 2007). The density of the RAW264.7 cells at the time of plating is critical for RANKL-induced osteoclast differentiation (Hayashi et al. 2008; Hotokezaka et al. 2002).

The first aim using this model was to observe the effects of RANKL stimulation and osteoclast differentiation on Hn1 expression. The second aim was to analyze whether silencing Hn1 with the Hn1-siRNAs could drive cell differentiation independent of RANKL stimulation. The hypothesis was derived from the B16.F10 experiments where Hn1 depletion induced p38 phosphorylation and subsequent cell differentiation.

The second model used was the HEK293 cell line. These were derived from primary human embryonic kidney (HEK) cells that were transformed with sheared adenovirus-5 (Graham et al. 1977). The protein expression pattern of HEK293 cells indicates a higher resemblance to neurons than to kidney cells. They express the neurofilament proteins NF-L, NF-M, NF-H, and α-internexin, as well as vimentin, certain neurotransmitters, neurotransmitter receptors, ion channels and other neuron-specific markers. Because they express high levels of NF-M, NF-L and vimentin, and low levels of NF-H and α-internexin, they are said to resemble early differentiating neurons (Shaw et al. 2002). Preliminary analyses indicated that these cells do not express HN1 protein. The purpose of this particular study was to examine the effects of overexpressing the human form of HN1 in HEK293 cells on the expression levels of neurofilaments NF-H, -L, -M and α-internexin. The expression patterns of these neurofilaments serve as indicators for different stages of neuronal differentiation because during development, α-internexin expression precedes NF-L and NF-M expression (McGraw et al. 2002; Shaw et al. 2002). NF-H expression appears last, and some fully differentiated neurons never express it.
Based on the results from previous experiments indicating that Hn1 may be able to maintain cells in a dedifferentiated state, the hypothesis for this experiment was that HN1 overexpression in HEK293 cells would cause a decrease in the levels of neuronal differentiation markers (NF-H, -M, and –L) and an increase in α-internexin, indicating a lower stage of differentiation.

Results

Effect of RANKL Treatment on Hn1 Expression in RAW264.7 Cells

Based on the knowledge that RAW264.7 cells differentiate into osteoclasts upon stimulation with RANKL, this first set of experiments focused on evaluating the correlation between Hn1 expression and differentiation in these cells. First, it was determined by Western blot analysis that RAW264.7 cells express Hn1 protein. Cells were then plated and treated with RANKL for five to seven days, until multinucleation and osteoclast differentiation were visible. Protein lysates were collected and analyzed by Western blot using the rabbit polyclonal Hn1, MITF, Tyrosinase, and actin antibodies (Figure 6-1). The results showed that tyrosinase expression was elevated while none of the other proteins analyzed changed upon RANKL stimulation. The activation of MITF was verified by the elevated expression of tyrosinase, a protein regulated by MITF.

Effects of Hn1 Depletion on Cell Differentiation

RAW264.7 cells were treated with AAV serotypes 2 and 6 expressing the Hn1- or scrambled-siRNA (Figure 6-2A). Hn1-siRNA-B in AAV2 was the most efficient at inhibiting Hn1 expression. In a second experiment, cells were treated with the scrambled and Hn1-siRNA and monitored by light microscopy on a daily basis for 7-10 days. There were no visible changes in the morphology or differentiation state of these cells as a consequence of Hn1 depletion (Figure 6-2B).
Effect of Hn1 Overexpression on Neuronal Markers of HEK293 Cells

HEK293 cells normally express high levels of neuronal markers NF-M and NF-L and low levels of NF-H and α-internexin, but no detectable HN1. The effect of overexpressing human HN1 on levels of HEK293-expressed neurofilaments were examined after transfection with a plasmid vector expressing human HN1, or the empty plasmid vector (control). Western blot analysis using antibodies specific to each neurofilament protein indicated that the most obvious change in HEK293 cells as a result of HN1 overexpression was increased α-internexin expression (Figure 6-4). Levels of NF-H, -M, and -L remained unchanged.

Discussion

These experiments were designed to explore the relationship of Hn1 and the differentiation state of two cell types, the murine RAW264.7 and human HEK293 cells. The first part of these experiments indicated that Hn1 protein was intrinsically expressed in RAW264.7 cells, but that RANKL-stimulated osteoclast differentiation did not affect Hn1 expression. Moreover, Hn1 depletion did not promote cell differentiation independent of RANKL. These data suggest that Hn1 does not play a role in the differentiation of osteoclasts from RAW264.7 cells. It has been reported that RANKL-induced differentiation occurs via MITF in these cells (Sharma et al. 2007). However, Hn1 depletion, unlike its apparent negative effect on MITF in B16.F10 cells, did not affect MITF levels in the RAW264.7 cells. These data further support a potential role for Hn1 in regulating differentiation via an MITF-independent mechanism.

The second part of this study focused on the effect of overexpressing human HN1 in the human HEK293 cell line. The neurofilaments that characterize these cells as neuron-like cells were evaluated and it was determined that α-internexin expression was elevated as a result of HN1 overexpression. This indicates that HEK293 cells may become slightly less differentiated.
as a result of Hn1 overexpression. Neurofilament proteins NF-H, -M, -L and α-internexin, as
well as nestin are all members of the intermediate filament family. During development of the
mammalian nervous system, α-internexin is expressed earlier than the other neurofilament
proteins (Kaplan et al. 1990). Characterization of neurofilaments in models of peripheral nerve
injury that lead to successful regeneration (e.g. facial motoneurons that undergo nerve crush or
transection), indicates that neurofilament proteins NF-H, -M and -L are downregulated after
injury (Goldstein et al. 1988; Tetzlaff et al. 1991; Tetzlaff et al. 1988), whereas α-internexin
expression increases dramatically by three days after injury and then disappears 28 days after
injury, which corresponds with full regeneration. However, expression of α-internexin remains
elevated in neurons that undergo resection and fail to regenerate after injury. Target
reinnervation results in cessation of α-internexin expression (McGraw et al. 2002). This is
consistent with the expression pattern of Hn1 in the adult facial nerve axotomy model, where
Hn1 expression, like α-internexin, is elevated by three days after nerve crush or transection,
reaches a peak at 5-7 days post injury, and returns to basal level by day 28, at which time
regeneration is complete. Like α-internexin, Hn1 mRNA levels were also sustained after nerve
resection (Zujovic et al. 2005). From these combined reports, it appears that α-internexin is
elevated when Hn1 is increased (McGraw et al. 2002; Zujovic et al. 2005). Alpha-internexin
expression is transiently increased 7 days after rubrospinal tractotomy and its expression lasts
about one week, however, these neurons do not regenerate. Hn1 is not expressed in this model
(McGraw et al. 2002; Zujovic et al. 2005). Alpha-internexin expression remains to be evaluated
in the neonatal facial nerve axotomy, another model of injury where neurons fail to regenerate
and where Hn1 expression does not increase (Zujovic et al. 2005). The expression pattern of α-
internexin seen in the HEK293 cells transfected with HN1 is consistent with these reports and together they suggest that HN1 overexpression may cause elevation of α-internexin expression.

Xefiltin and gefiltin, the homologs of α-internexin in amphibians and fish, are also upregulated during development of optic axons and again during their regeneration after injury. The factors that regulate these proteins are likely soluble and come from the tectum (Asch et al. 1998; Glasgow et al. 1994; Niloff et al. 1998; Zhao and Szaro 1997a; Zhao and Szaro 1997b). This is consistent with the high expression of Hn1 during retinal regeneration of the Japanese common newt (Goto et al. 2006). Because gefiltin as well as a homolog of Hn1 exist in zebrafish, it would be interesting to monitor the effect of Hn1 depletion on optic nerve regeneration in the zebrafish.

This study provides evidence that HN1 overexpression regulates α-internexin expression. It has been suggested that because the increase in α-internexin during rodent nerve regeneration is not seen when blocking retrograde transport, it is possible that an inhibitory signal from the periphery or the muscle that it innervates inhibits the expression of α-internexin when neurons are not injured, and that when they are, the inhibitory signal is removed, allowing the expression of α-internexin (McGraw et al. 2002). An interesting direction to investigate would be to elucidate how HN1 may control the upregulation of α-internexin.
**Figure 6-1.** Hn1 expression in RAW264.7 cells treated with and without RANKL. RAW264.7 cells were treated with or without RANKL for 5 days, at which time the RANKL-treated cells had formed osteoclasts. Hn1 expression was evaluated in these cells by Western blot using the polyclonal Hn1 antibody. Tyrosinase expression was evaluated as a downstream effect of MITF transcriptional activation.
Figure 6-2. Effect of Hn1 depletion on RAW264.7 differentiation. A) RAW264.7 cells were treated with AAV6 expressing scrambled- or Hn1-siRNA or AAV2 expressing Hn1-siRNA-B, or no virus and analyzed by Western blot. Hn1-siRNA-B in AAV2 was the most efficient at depleting Hn1 protein in these cells. B) Light microscopy of RAW264.7 cells treated with scrambled- or Hn1-siRNA-B AAV2.
Figure 6-3. Effect of HN1 overexpression on HEK293 neurofilaments. Western blot shows the expression of four neurofilaments (NF-H, -M, -L and α-internexin) in HEK293 cells three days after transfection with either control- or recombinant human HN1-expressing plasmids.
CHAPTER 7
GENERAL DISCUSSION

Summary of the Main Findings

As more information about Hn1 is discovered, it becomes more clear that this is an important gene to study. Its high expression in development, plastic regions of the brain, regenerating neural tissue, and cancer suggests that it plays an important role in the regulation of cell differentiation and plasticity. The first of the main findings in this study was the identification of Hn1 protein expression in numerous cancer cells and tissues. Hn1 is expressed not only in murine models of very aggressive cancers, namely the B16.F10 melanoma and the GL261 glioma, but also in human high-grade glioma tissue as well as numerous human cancer cell lines including esophageal squamous cell carcinoma, ovarian carcinoma and erythroleukemia. Studies conducted in other laboratories indicate that HN1 is also present in human melanoma and esophageal carcinoma tissues as well as breast cancer cell lines.

Secondly, Hn1-expressing GL261 cells formed significantly larger tumors in vivo than Hn1-depleted GL261 cells, even though Hn1 expression did not affect the growth of these cells in vitro. However, the in vitro growth rate of Hn1-expressing B16.F10 cells was faster than that of Hn1-depleted cells. Why the expression of Hn1 affects the in vitro growth rates of these two cancer cell lines differently is unknown, but one possibility involves the high interaction of GL261 tumors with their environment. As they grow, GL261 gliomas elicit a strong immune response from the surrounding tissue, whereas B16.F10 melanomas are poorly immunogenic (Peter et al., 2001; Seliger et al. 2001).

Another main finding, using the B16.F10 melanoma cell line, was the discovery that Hn1 is likely to play a role in the regulation of cell differentiation. The increased melanogenesis and arrest of the cell cycle consequent to Hn1 depletion are phenotypical of a more differentiated
melanocyte. The data presented provide evidence that protein expression changes took place in the B16.F10 cells consequent to Hn1 depletion. These changes included elevated expression of melanogenic enzymes, increased protein interactions that regulate the secretion of melanin, changes in cell cycle and growth regulatory proteins including increased p21 and cyclin D1 expression, reduced p27 and c-Met expression, Rb and ERK hypophosphorylation and p38 hyperphosphorylation. The unexpected downregulation of MITF remains to be explained. However, the Hn1-dependent effects on the differentiation of these cells appear to be independent of either MITF or USF-1. Alternative mechanisms, including transcription factors TFE3 and Sox10 should be explored.

Finally, the relationship between Hn1 and cell differentiation was further investigated in RAW264.7 and HEK293 cells. There was no detected relationship between differentiation of osteoclasts from RAW264.7 cells and Hn1. However, Hn1 overexpression in HEK293 cells caused the upregulation of α-internexin, a neurofilament that is developmentally expressed before all others and whose expression is also induced in regenerating models of nerve injury. This was especially interesting considering the previously reported upregulation in Hn1 expression in models of neuronal regeneration in rodents and newts (McGraw et al. 2002; Zujovic et al. 2005).

**Shortcomings of Experiments in Hindsight**

The immunoprecipitation analyses conducted on both GL261 and B16.F10 cells indicated that Hn1 binds actin. However, because of the lack of colocalization of these two proteins as seen by confocal microscopy and the high nuclear localization of Hn1 in these cells, this interaction between actin and Hn1 seems unlikely. It would be of interest to further investigate this interaction in the intracellular environment. More importantly, the technical approach of the
immunoprecipitation experiment needs to be addressed, since identifying the proteins that interact physically with Hn1 is highly important.

The experiments conducted in the RAW264.7 cells were technically difficult and limiting. Originally, the goals included analyzing the intracellular localization of Hn1 and the effect of Hn1 depletion on RANKL-driven differentiation. However, the ability to cause the cells to differentiate with RANKL was inconsistent. Multiple approaches including the reported optimal cell densities and methods (Hayashi et al. 2008; Hotokezaka et al. 2002) were tested, but the RANKL-driven differentiation appeared to occur sporadically. It has been reported that the differentiation of RAW264.7 cells is a difficult task to accomplish. A group has identified and isolated three RAW264.7-derived clonal cell lines that generate 100% osteoclast formation with RANKL treatment. It may be useful to analyze the effects of Hn1 on these cells, since their differentiation is more likely than that of parental RAW264.7 cells (Cuetara et al. 2006). The variability of these is extended by a report on the non-uniform response of RAW264.7 cells to bacterial infection (Turco and Winkler 1982).

**Future Directions**

After investigating the expression and function of Hn1 in several cancers and the possibility of its involvement in maintaining cells in a dedifferentiated state, many questions arise. These questions should be addressed to elucidate the exact role and mechanism of this widely acting protein. Below is a series of questions, hypotheses and experiments that are proposed for the next investigator who assumes the exciting challenge of discovering the mechanism of Hn1.

**Can Hn1 Overexpression Cause a Differentiated Cell to Dedifferentiate?**

Hn1 overexpression on HEK293 cells caused upregulation of α-internexin expression. However, these cells may not be the ideal models to verify whether Hn1 can really induce cell
dedifferentiation. A more interesting and informative experiment to test whether Hn1 is promoting the dedifferentiation of cells would be to allow primary neuronal cells in culture to differentiate and then transduce them with a neuron-specific viral vector expressing Hn1. If Hn1 can cause a cell to become dedifferentiated, these neurons should re-enter the cell cycle and they should stop expressing differentiated neuronal markers, such as NF-H, -M and -L, as they begin to express developmental neuronal markers such as α-internexin or even vimentin and nestin, which are seen earlier in development. They should also cease to have action potentials, measurable by electrophysiology. Positive results from this experiment would not only prove that Hn1 is able to induce the dedifferentiation of a differentiated neuron, but it would also provide a link between the role of Hn1 in cancer and neural regeneration.

An alternative would be to use rat PC12 cells, which differentiate into neuron-like cells after treatment with nerve growth factor (NGF). These cells could be cultured and treated with NGF as well as Hn1 to determine if they still differentiate into neurons in the presence of Hn1 or if Hn1 expression inhibits their differentiation.

Is Hn1 Depletion Sufficient to Cure Cancer?

Regarding the GL261 experiment where Hn1-expressing cells form larger tumors than Hn1-depleted cells, it would be valuable to test a similar but more clinically relevant hypothesis. Can the growth of normal Hn1-expressing GL261 tumors be arrested by treating with Hn1-siRNA in vivo? Since the tumors would be established without prior treatment with the Hn1-siRNA, this experiment would require the construction of a specific viral vector that will be able to access and target the tumor cells to deliver Hn1-siRNA in vivo. Successful treatment of the tumor with such viral vector would show whether or not treatment with the Hn1-siRNA is sufficient to cure gliomas by arresting their growth and prolonging the survival of the tumor-
bearing animals. This viral vector must be specific not only to target the cancer cells but also to
spread among the surrounding cancer cells and not the healthy surrounding tissue. Additionally,
these experiments could be conducted in B16.F10 skin tumors.

**Does Hn1 Depletion Increase Susceptibility to Chemotherapeutic Treatment?**

Preliminary experiments using chemotherapeutic agents 5-fluorouracil and VP-16 on
B16.F10 cells with and without Hn1 did not show any obvious effect of Hn1-depletion on
chemotherapeutic agent-driven apoptosis. However, further experimentation with a diversity of
chemotherapeutic agents is suggested to determine whether Hn1 depletion can render cancer
cells more or less susceptible to chemotherapeutic treatment. This knowledge could be useful if
Hn1 depletion itself is not enough to arrest tumor growth.

**Are the Effects of Hn1-depletion on Melanoma Cell Differentiation Really Independent of
MITF?**

To verify whether MITF really is not involved in the Hn1-depletion-dependent
differentiation pathway, B16.F10 cells can be treated with the Hn1- and scrambled-siRNAs, and
subsequently with a scrambled- and MITF-siRNA. If MITF expression were efficiently knocked
down, and the Hn1-siRNA still had its reported effects on tyrosinase, Trp2, p38 and ERK
phosphorylation, and the cell cycle arrest, then it can be concluded that MITF is not involved in
the Hn1-depletion-driven differentiation of these cells. However, if these effects were no longer
viewed in the absence of MITF, it would indicate that MITF, although undetected by Western
blot analysis, is involved in this pathway in some form.

A complementary approach would be to conduct an RT-PCR of MITF from cells treated
with and without the Hn1-siRNA to determine if Hn1 depletion causes decreased MITF
transcription or if it causes the protein to be degraded or modified post-translationally.
Is Hn1-depletion Affecting Other Known Transcription Factors to Impact Melanoma Cell Differentiation?

As previously mentioned, transcription factors Sox10 and TFE3 can activate tyrosinase expression in melanocytes. They sometimes act in conjunction with MITF, but not always. The effect of Hn1 depletion on Sox10 as well as the potential binding of Hn1 to TFE3 may prove informative. Western blot analyses using antibodies against Sox10 on Hn1-expressing and Hn1-depleted B16.F10 cell lysates, as well as a co-immunoprecipitation analysis using antibodies against Hn1 and TFE3, would be beneficial in determining whether Hn1 has an effect on the expression or activity of these transcription factors.

Does Hn1 Directly Prevent p38 Phosphorylation?

As seen in the B16.F10 cell experiments, Hn1 depletion causes p38 phosphorylation to increase dramatically. It is also known that p38 phosphorylation promotes the apoptosis and degeneration of neurons that undergo injury. Can Hn1 bind and inhibit activators of p38 phosphorylation, such as MKK4, to prevent apoptosis after axotomy and allow for neuronal regeneration? A co-immunoprecipitation analysis of these two proteins may be helpful to determine the molecular mechanism that links Hn1 and p38.

Is Hn1 Overexpression Sufficient to Regenerate a Neuronal Axon? And Can Hn1 Depletion Hinder its Regeneration?

The neurons of the marine mollusk *Aplysia californica* are very large and relatively easy to culture for live imaging. When maintained *in vitro*, one neuron can be axotomized individually. This causes the formation of growth cones on both ends of the transected axon, subsequent growth and successful regeneration (Benbassat and Spira 1993). Labeling for Hn1 in these neurons may provide interesting information in relation to other proteins and events associated with axonal regeneration (Sahly et al. 2006). It could be hypothesized that, as seen in newts and rodents, Hn1 expression will increase during the growth of the axon and return to basal levels
once the axon has successfully regenerated. Even more interesting would be to see the effect of Hn1-siRNA expression in these cells. Would the transected axon still extend and consequently regenerate?

Another experiment to test whether Hn1 is necessary and sufficient for neural regeneration using rodent models would be to overexpress Hn1 in the neonatal facial motor neurons and see if Hn1 expression is sufficient to drive the regeneration of these cells after axotomy, as they do in the adult axotomy model. This would require a viral vector that is able to spread among the neurons in the facial motor nucleus.

Additionally, it may be helpful to determine whether Hn1 is expressed in the olfactory bulb. Because of the highly regenerative nature of the neurons in the olfactory bulb, it is expected that Hn1 would be expressed in these neurons. Immunohistochemical analysis of this tissue would provide a clear answer.

**Is Hn1 Necessary for Memory Formation?**

The apparent role of Hn1 in cell plasticity and the high level of Hn1 mRNA in the hippocampus, cortex and cerebellum of the adult rodent brain suggest that Hn1 may play a role in learning and the formation of memories. There is evidence that ERK activation is necessary for long-term potentiation, the cellular basis for long-term memory, and that p38 activity is necessary for long-term depression. As shown in the B16.F10 experiments, Hn1 can affect these MAP kinases in such a way that would correlate with memory retention. This is a valid hypothesis that could be tested by behavioral analysis of the performance of mice with and without functional Hn1 in a Morris water maze. This would require Hn1 inhibitors, yet to be determined, to treat the rodents, or Hn1-knockout rodents, if they are able to survive development without Hn1.
What is the Developmental Phenotype of an Hn1 Knockout Transgenic Mouse or a Jupiter Knockout Drosophila melanogaster?

A fundamental question is whether animals are still able to develop and survive without Hn1 protein. This would be directly addressed by creating a transgenic Hn1-knockout mouse. An alternative would be either an Hn1-knockout zebrafish or a Jupiter-knockout D. melanogaster. If these can be created and Hn1 is not required for survival, what are the developmental consequences, if any? These would also be very valuable tools for further studies about Hn1.

Is There a Correlation Between Hn1 and Neurodegenerative Diseases?

There are several reasons to consider this hypothesis. One is that Hn1 is clearly involved in proper motor nerve and retinal regeneration. Neurodegenerative diseases that fall in this category include amyotrophic lateral sclerosis (ALS) and macular degeneration, which causes blindness. Also, the downregulation of cell pigmentation by Hn1 in the B16.F10 cells may suggest that Hn1 is normally absent from the dopaminergic neurons of the substantia nigra, which are highly pigmented. The degeneration of these neurons is the cause of Parkinson’s disease. Is it possible that Hn1 expression in these cells could prevent these neurons from degenerating? Moreover, there is evidence of abnormal accumulation of neurofilaments in neurodegenerative disorders including ALS and Parkinson’s disease (Lariviere and Julien 2004). The increased expression of α-internexin seen in HEK293 cells consequent to Hn1 overexpression may indicate that Hn1 is driving its expression, which could be necessary to stabilize neurofilaments in the axon. Analysis of Hn1 protein expression in brain and retinal tissues from patients with a diversity of neurodegenerative diseases may provide insight into the relevance of this protein in these different scenarios.
Is Hn1 a Transcription Factor?

The experiments presented in this study show that Hn1 depletion and overexpression cause a wide array of intracellular changes. Hn1 expression has an effect on the expression or phosphorylation of different proteins. One possibility is that Hn1 is a transcription factor acting to regulate the expression of multiple factors that influence the cell in multiple ways. To determine this, a microarray analysis in cells with and without Hn1 may identify what genes change. Chromatin immunoprecipitation analyses may be useful in identifying whether Hn1 binds DNA. If Hn1 were a transcription factor, it would explain why Hn1 depletion does not cause an upregulation of MITF or USF-1 despite the cell cycle being effectively arrested and melanogenesis elevated.

What Factors Control the Expression and Activity of Hn1?

Identifying the promoter that activates Hn1 expression could provide immense insight into its function. Promoter-binding motifs in the genomic DNA sequence upstream of its first exon could help identify potential transcription factors that may regulate Hn1 expression. The transcription factors with most Hn1-regulating potential, i.e. those that are known to have a role during development, can be tested experimentally to determine whether they regulate Hn1 expression. Discovery of the Hn1 promoter may also provide clues about the upstream cellular factors that cause its expression or repression.

Aside from the regulation of Hn1 expression, it may be helpful to identify the factors that regulate its activity. As mentioned in the introduction, it has been shown that Hn1 undergoes phosphorylation during the mitotic phase of the cell cycle. An immunoprecipitation analysis of Hn1 in cells arrested at the mitotic phase may provide insight on the significance of this phenomenon, if different proteins can bind Hn1 in different phosphorylation states.
How Can HN1 be Used as a Target for Therapies Against Cancer and Neurodegenerative Diseases in Humans?

Part of determining the answer to this question will be in conducting the experiments described above. However, if results show that an anti-cancer gene therapy targeting HN1 protein really is pursuable, it will be necessary to engineer and build a siRNA targeted to degrade the human HN1 mRNA. Likewise, if HN1 is also sufficient to promote neuronal regeneration or to prevent degeneration, it will be beneficial to develop an HN1-expressing vector to be used in gene therapy against neurodegenerative diseases. Great caution must be taken while doing this to prevent unforeseen effects on humans, since viral vectors may have the ability to integrate into the organism’s genomic DNA. The development of an alternative pharmaceutical approach to treat these conditions may be possible once the factors that regulate Hn1 expression and activity are identified.

Concluding Remarks

In vertebrate embryos, the neural crest gives rise to neurons, glia, melanocytes, bone, cartilage, connective tissue, smooth muscle and endocrine cells. Moreover, once differentiated, these cells of neural crest origin have the ability to reverse to a multipotent state and trans-differentiate in vitro (Le Douarin and Dupin 2003). Coupled with reports on Hn1 expression throughout multiple tissues during development, including brain, spinal cord, spleen, heart, thymus and testis (Zujovic et al. 2005), it is possible that Hn1 protein is expressed exclusively by cells of neural crest origin. It is also possible that Hn1 may be the protein providing these cells with the plasticity necessary for regression to multipotent stages and for regeneration. However, this fails to explain why some neurons (the ones that do not regenerate) do not express Hn1.

Especially interesting in these experiments is the increase in p38 phosphorylation and decrease in ERK phosphorylation as a consequence of Hn1 depletion in the B16.F10 cells. ERK
and p38 are not only implicated in cancer growth and cellular differentiation. They are also
expressed in areas of the brain involved in learning and memory and are necessary for long-term
potentiation and depression, which are the mechanisms involved in memory retention. Also,
ERK1 expression is increased in vagal neurons 7 days after axotomy and its expression decays
by 28 days, which is consistent with Hn1 and α-internexin expression in models that regenerate
including axotomy of the adult facial motor and vagal nerves. Perhaps one of the most interesting
points to focus on in the future is the positive correlation between Hn1 and α-internexin
expression and ERK phosphorylation.

Perhaps a better way to reach an understanding on the function and mechanism of Hn1 is
by first asking, what controls Hn1? Besides the information that would be provided by
identifying its promoter, we know that Hn1 expression is induced by nerve axotomy. Is it
induced by stress or by the intracellular calcium elevation resulting from damage to protect from
apoptosis? Probably not, because Hn1 is also highly expressed in multiple tissues during
development and also in healthy hippocampal and cortical neurons, where cells divide and grow
or simply can remodel their structure for plasticity, but are not necessarily stressed or damaged.
At what point during development is Hn1 expression first induced? Maybe it is expressed from
the start. This could be a possibility since its expression is found in testis. If this is the case,
another factor must downregulate its expression after development in order to induce cellular
differentiation. Whatever factor inhibits Hn1 expression must be absent in cancers. It must also
be downregulated as a result of axotomy and other injuries to nervous system where retrograde
signals within the neuron are still possible. Is it a protein from the target tissue such as the
innervated muscle of the motoneuron or the tectum of eye? This molecule would also be absent
from neurons in areas of high plasticity in the brain. Changing the way we think about Hn1 may lead us into a more clear direction for future experiments.

Many more years must be dedicated to the study of this promising protein of interesting characteristics. Its therapeutic potential is enormous, since it is involved in a very wide array of biological events from development, to plasticity, to regeneration to cancer. The factors that are regulated by Hn1 as well as the factors that regulate it must be defined in order to understand the complete functions of this protein and to be able to use it as a target for therapies against cancer and neurodegenerative diseases.
APPENDIX A
IDENTIFICATION OF PROTEINS THAT INTERACT WITH HN1

To gain insight into the function of Hn1 globally, a series of immunoprecipitation (Zolotukhin et al.) analyses were conducted in the GL261 and B16.F10 cells. The first experiment was done on GL261 cell lysates using the rabbit polyclonal anti-Hn1 antibody. As the negative control for the immunoprecipitation, a non-immune rabbit IgG was used. Silver staining of SDS-PAGE samples from the immunoprecipitates revealed three prominent bands in the reaction containing the anti-Hn1 antibody that were not present in the precipitates derived from the non-immune IgG reaction (Figure A1). An identical gel was stained with colloidal Coomassie dye and the three corresponding bands were isolated and subjected to mass spectrometry analysis. Actin, myosin and tropomyosin were identified as the proteins that more abundantly co-precipitated with the Hn1 antibody.

To confirm the interaction between actin and Hn1, further analysis was conducted on B16.F10 as well as GL261 cells using either rabbit polyclonal antibodies against actin or Hn1 or rabbit non-immune IgG (Figure A2). The final pellets from the IP reactions were analyzed by Western blot using mouse monoclonal antibodies against Hn1 and actin. Hn1 and actin coprecipitated in both cell types. In the B16.F10 cells, the levels of Hn1 and actin that coprecipitated with the non-immune rabbit IgG were significantly lower than the coprecipitates using the specific antibodies. However, similar levels of actin coprecipitated with the non-immune IgG in the Gl261 cells.

Furthermore, immunocytochemical and confocal analysis of B16.F10 cells using the polyclonal anti-Hn1 antibody and phalloidin did not show colocalization of Hn1 and actin (Figure A3). Therefore, the data were inconsistent with the results of the co-immunoprecipitation analyses. Hn1 was found throughout the cytoplasm, but more prominently in the cell nuclei,
whereas the cytoskeletal protein actin was found in the cytoplasm and not the cell nuclei. Because the process of preparing the cell lysates mixes proteins that are normally compartmentalized in the nucleus or cytoplasm of the cells, it is possible that the interaction between actin and Hn1 does not occur in a natural environment.

Although the interaction of Hn1 and actin proposed in these experiments is not yet confirmed, identifying proteins that interact with Hn1 would provide more clarity about the molecular mechanism of this protein. As discussed in the introduction, human HN1 has been reported to have a physical association with Rho-type GTPase-activator protein RICH2 and with splicing factor proline- and glutamine-rich (SFPQ) (Stelzl et al. 2005). This interaction must be confirmed by co-immunoprecipitation and intracellular colocalization analyses. Both Hn1 and SFPQ are highly expressed in the brain during embryonic development and in adult hippocampus, cortex and cerebellum (Chanas-Sacre et al. 1999; Zujovic et al. 2005). RICH2 mRNA is also abundant in human brain (Richnau and Aspenstrom 2001). Like Hn1, SFPQ and RICH2 are involved in the regulation of neuronal development, survival and neuronal differentiation (Lowery et al. 2007). RICH2 activates Rho-GTPases (Rac1 and Cdc42) that are also involved in neuronal regeneration after axotomy in mice (Tanabe et al. 2003). These Rho-GTPases are overexpressed in several human tumors and are involved in differentiation, survival and cell cycle progression (Fritz et al. 1999; Fritz and Kaina 2006). SFPQ is also commonly seen in papillary renal cell carcinoma. The fusion of SFPQ and the transcription factor TFE3 in healthy renal epithelial cells leads to dedifferentiation (Mathur and Samuels 2007). Can binding to HN1 cause RICH2 or SFPQ to allow growth of cells during cancer, development and regeneration? Identifying the binding partners of Hn1 is a sensible step to pursue in order to delineate the intracellular pathway and molecular function of action of this protein.
Figure A-1. Identification of Hn1-binding proteins in GL261 cells. Silver stained gel of an immunoprecipitation analysis of GL261 protein lysates using the rabbit polyclonal anti-Hn1 antibody (Ab) and non-immune rabbit IgG as the control. Not shown is an identical non-silver stained gel was stained with colloidal Coomassie dye and prominent bands were isolated and subjected to mass spectrometry analysis. The proteins that were identified to co-immunoprecipitate with the anti-Hn1 antibody were actin, myosin 9, and tropomyosin.
Figure A-2. Association of Hn1 and actin in B16.F10 and GL261 cells. Immunoprecipitation analyses were conducted on both B16.F10 and GL261 cells using either rabbit polyclonal antibodies against Hn1 or actin or a non-immune IgG as the control. Co-precipitates were analyzed by Western blot using mouse monoclonal anti-Hn1 and anti-actin antibodies.
Figure A-3. Immunohistochemical analysis of Hn1 and actin. B16.F10 cells were plated on glass coverslips and subjected to immunocytochemical analysis using the Hn1 antibody (red). Intracellular actin was also stained with phalloidin (Greene et al.), and cell nuclei were stained with DAPI (blue).
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BIOGRAPHICAL SKETCH

Katharine Laughlin was born in Caracas, Venezuela on March of 1982 to Barry and Malena Laughlin. She attended the school Colegio Jefferson in Caracas until 1995, when she immigrated to the United States of America with her parents and brother. She graduated from Coral Springs High School, Florida in 2000. Four years later, she obtained her Bachelor of Science degree in cellular and molecular neurobiology from the University of Florida. Her undergraduate thesis research “Optimizing the control of gene expression provided by the tetracycline-regulatable system for gene therapies” was conducted in the laboratory of Dr. Susan Semple-Rowland under her mentorship. In August of 2004, Kathy began her graduate studies at the University of Florida College of Medicine Interdisciplinary Program (IDP) in Biomedical Science. She did rotations in the laboratories of Dr. Jeffrey K. Harrison and Dr. Edwin Meyer in the Department of Pharmacology and Therapeutics, and Dr. Leonid Moroz in the Department of Neuroscience at the Whitney Marine Laboratory in Marineland, Florida. She joined the laboratory of Dr. Harrison in May of 2005.