PERLECAN REGULATION OF SONIC HEDGEHOG SIGNALING IN ADVANCED PROSTATE CANCER: FROM *DROSOPHILA* TO HUMANS

A Dissertation

by

ANA MARIA HERNANDEZ COTES

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2008

Major Subject: Biochemistry

PERLECAN REGULATION OF SONIC HEDGEHOG SIGNALING IN ADVANCED PROSTATE CANCER: FROM *DROSOPHILA* TO HUMANS

A Dissertation

by

ANA MARIA HERNANDEZ COTES

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Approved by:

Chair of Committee,	Sumana Datta
Committee Members,	Wallace McKeehan
	Jean Phillipe Pellois
	Vladislav Panin
	Bruce Riley
Head of Department,	Gregory Reinhart

May 2008

Major Subject: Biochemistry

ABSTRACT

PERLECAN Regulation of SONIC HEDGEHOG Signaling in Advanced Prostate Cancer: From *Drosophila* to Humans.

(May 2008)

Ana Maria Hernandez Cotes, B.S., Universidad de Los Andes

Chair of Advisory Committee: Dr. Sumana Datta

Prostate cancer is the second leading cause of death from cancer in men in the United States. Most men will die of the advanced, metastatic form of the disease. Thus, treatment strategies targeting the metastatic form of the disease are especially needed. Emerging research on metastatic cancer highlights the importance of the microenvironment in cancer progression and metastasis, with an emphasis on deregulated developmental signaling in cancer progression. Research in model organisms has shown that developmental signaling pathways are regulated by various components of the extracellular matrix, including heparan sulfate proteoglycans. In the model system Drosophila, the heparan sulfate proteoglycan Trol is needed for Hh-dependent proliferation in quiescent neural stem cells. In collaboration with others, I have shown that the human homolog of Trol, PERLECAN, regulates SONIC HEDGEHOG-dependent proliferation in advanced prostate cancer by two different mechanisms. This makes PERLECAN a potential drug target and biomarker for prostate cancer screening and treatment. My results also validate the discoveries made in Drosophila in the context of human disease. With this validation, I propose and describe the Drosophila Ejaculatory Bulb (EjB) as model for prostate cancer and prostate aging.

DEDICATION

To my family, whose enthusiasm and support have been essential for this endeavor. To my parents and aunt for their love and endless encouragement and to my brother Amaury, for his truly unconditional love and understanding.

ACKNOWLEDGEMENTS

First and foremost I would like to thank Dr. Sumana Datta. I could not have asked for a better mentor during my graduate work. Her enthusiasm and support as well as her profound insight have been invaluable to me. I thank my committee, Dr. Bruce Riley, Dr. Vlad Panin, Dr. Wallace McKeehan, Dr. Patricia LiWang and Dr. Jean-Philippe Pellois for their helpful advice and guidance throughout my graduate career. I want to thank the members of the Datta lab, especially Dr. Andrea Barrett, for her help, support and friendship. My thanks go to Brent Ferguson and Jonathan Lindner for their generous help with teaching and research. Lastly I want to thank all of my friends at Texas A&M who have made this an unforgettable experience.

TABLE OF CONTENTS

CHAPTER Page

LIST OF FIGURES

FIGURE Page

LIST OF TABLES

CHAPTER I
INTRODUCTION

The mechanistic analysis of complex diseases such as cancer requires the integration of multiple approaches and paradigms. It has been known for several years that the accumulation of genetic mutations resulting in the activation of oncogenes and the inactivation of tumor suppressors is the most probable cause of cancer initiation. We now suspect that these mutations accumulate in a unique population of cells called stem cells and result in unrestrained proliferation. Yet uncontrolled proliferation and decreased apoptosis alone do not lead to the massive tissue rearrangements, neovascularization and metastasis that characterize cancer. These traits of malignant neoplasia arise when the environment surrounding the precancerous lesion is altered, and its precise signaling cues go awry. Recent investigations have uncovered the role of misregulated developmental signaling in promoting metastatic behavior in a number of cancers. Many of these signaling pathways (such as Sonic Hedgehog or Fibroblast Growth Factor) rely heavily on the extracellular matrix (ECM), a dynamic three-dimensional network of fibrous proteins and proteoglycans such as Perlecan and Syndecan, to relay their messages to the receiving cells. The extracellular matrix is a dynamic environment and changes in its composition have a great impact on developmental signaling. Thus the study of ECM alterations during aging or the development of malignancy will help us to better understand the mechanisms needed for progression to metastasis, as well as provide us with a better basis for the development of treatment strategies.

This dissertation follows the style and format of Developmental Biology.

Emerging paradigms in cancer biology

The analysis of the mechanisms underlying the onset of advanced cancer is often obscured by the heterogeneous nature of the disease; morphology, inmunophenotype, and genotype vary enormously between patients (Shah et al., 2004). Thus, the disease we commonly call "cancer" comprises an enormous number of different pathologies, with different causes and mechanisms of development. This increases the already difficult task of targeting cancer cells and preventing them from multiplying and metastasizing while keeping normal cells functional.

The classical approach to understanding cancer biology involves the study of cancer cells both at the genetic level and at the level of cell behavior, to determine the characteristics in which they differ from their normal counterparts. Thus, studying cells from tumors with different degrees of malignity provides clues as to which events came earlier in the progression of cancer. Another part of this approach includes genetic studies of families with a history of recurring cancer, in an effort to discover mutations in specific genes that can be linked to a higher risk of developing a specific type of cancer.

Initially the genes linked with cancer were classified as oncogenes or tumor suppressors, based on the phenotype they related to. The term oncogene initially referred to a viral gene capable of generating neoplastic proliferation in cultured cells (Collier and Largaespada, 2006). Later, the term proto-oncogene would be coined for a human gene in which misactivating mutations would cause neoplastic transformation, or that caused a dominant cancer phenotype in a familial cancer syndrome. Following this scheme, tumor suppressor genes were defined as genes in which inactivating mutations would cause neoplastic transformation, or that caused a recessive cancer phenotype in syndromes of familial neoplasia. Thus proto-oncogenes such as Ras and c-myc, as well as the important tumor suppressors Rb and p53 were identified (Collier and

Largaespada, 2006). Further studies identified many of these genes as key components of intracellular signaling pathways that controlled processes such as cell cycle arrest and apoptosis (reviewed in (Kopnin, 2000)), thus providing the first mechanistic clues to the nature of the disease.

While this paradigm has been crucial in identifying many of the principal mutations that lead to cancer and tumorigenesis, some questions remain. For example, Transfrormigng Growth Factor-β (TGF-β) has been shown to have tumor suppressor effects in early carcinogenesis, yet its upregulation in advanced cancer correlates with poor prognosis (Wakefield and Roberts, 2002). In order to reconcile this apparently conflicting data we can use approaches that take into account the broader context in which these genes interact. Instead as thinking about cancer cells as independent entities, we can look at the tumor as an integrated system in which communication between cells and their microenvironment is as necessary for tumor progression as oncogenic mutations in individual cells. In this context it becomes important to understand and elucidate how these genes act together in signaling pathways, how these pathways are regulated by the extracellular matrix components, and how different pathways can act in a combinatorial manner to affect cancer progression in ways that are tissue-specific, and context-dependent.

Currently, we have a detailed picture of how the accumulation of mutations throughout a cell's lifetime leads to genomic instability, misregulated intracellular signal transduction, and ultimately a cancer phenotype. However, we do not know very much about the way the tumor coordinates its own development as a whole, as well as the interactions with the surrounding tissue that ultimately result in angiogenesis and metastasis. Understanding these processes is essential in developing treatments that target more aggressive forms of cancer, which currently have the poorest prognoses.

The cancer stem cell theory

One of the more pressing problems in cancer treatment is that complete tumor eradication seems to be needed in order to have tumor remission. If a few cells are missed during surgery, or become insensitive to radio/chemotherapy, the tumor can reappear, frequently with a more aggressive phenotype and reduced sensitivity to treatment agents (Pardal et al., 2003). Finding out how a whole tumor originates from a single cancer cell has been a subject of study for several years.

Analysis of different types of carcinomas, especially cancers of the hematopoietic system, has unveiled certain characteristics that gave rise to the idea that cancer cells shared common characteristics with stem cells, namely proliferative potential and multipotency. This has lead to the application of the principles and paradigms used in stem cell and developmental biology as a way of understanding cancer progression.

The discovery that a single hematopoietic stem cell could reconstitute bone marrow in an X-ray irradiated mouse (reviewed in (Huntly and Gilliland, 2005)) launched the field of stem cell biology, over 50 years ago. A stem cell is now defined as a cell capable of self-renewal and pluripotency. Since the discovery of hematopoietic stem cells, stem cells have been identified in other organs of the adult human, including neural stem cells and breast stem cells (Huntly and Gilliland, 2005).

Parallel to the discovery and further characterization of stem cells, it was shown that cancer cells originating from one tumor differed in their potential to form new growths; only a small percentage of the cells present in a tumor could actually give rise to a new tumor in vitro (Park et al., 1971), or when transplanted into a different host. This low clonogenic potential of cancer cells could be explained in two ways: 1) different populations of cells within a tumor may have different proliferation potentials, Or 2) all cells have the same proliferation potential, but only a few would find appropriate

environmental conditions to proliferate and form new growth. While these hypotheses do not necessarily exclude each other, formal demonstration of different populations of cells within a cancer with different proliferative potentials came in the form of experiments by Dick (Bonnet and Dick, 1997) and Kornblum (Hemmati et al., 2003).

In his studies of Acute Myeloid Leukemia, Dick isolated cell types based on immunological cell surface marker expression, and showed that only cells with a specific surface marker expression pattern had the ability to recapitulate the original disease when transplanted into host mice. Kornblum's experiments in pediatric brain tumors showed that cells isolated based on their capacity to form neurospheres could proliferate and differentiate into glial and neuronal lineages. These tumor-derived cells were more long-lived, and showed abnormal phenotypes consistent with the original tumor.

While the existence of stem cell-like populations in some cancers has now been clarified, the events that lead to cancer stem cell (trans)formation remain obscure; normal stem cells can acquire mutations resulting in transformation, or transformed precancerous cells may later acquire stem cell–like characteristics (Huntly et al., 2004)(Figure 1.1). These two events could even lead to different populations of cancer stem cells in the same disease. Further study of the chronological events taking place in the development of cancer stem cells is needed to distinguish between these two models. It is also necessary to investigate the events and pathways that regulate cancer stem cell self-renewal. To this end, previous studies normal stem cell biology to discover regulators of stem cell proliferation will prove of great value.

Thus, a paradigm emerges in which, within a specific tumor, only a small percentage of cells with stem cell-like characteristics are capable of reinitiating tumor growth (Figure 1.2). Since metastasis requires tumor initiation in a different part of the body, by definition, metastases have to be caused by dissemination of cancer stem cells. This

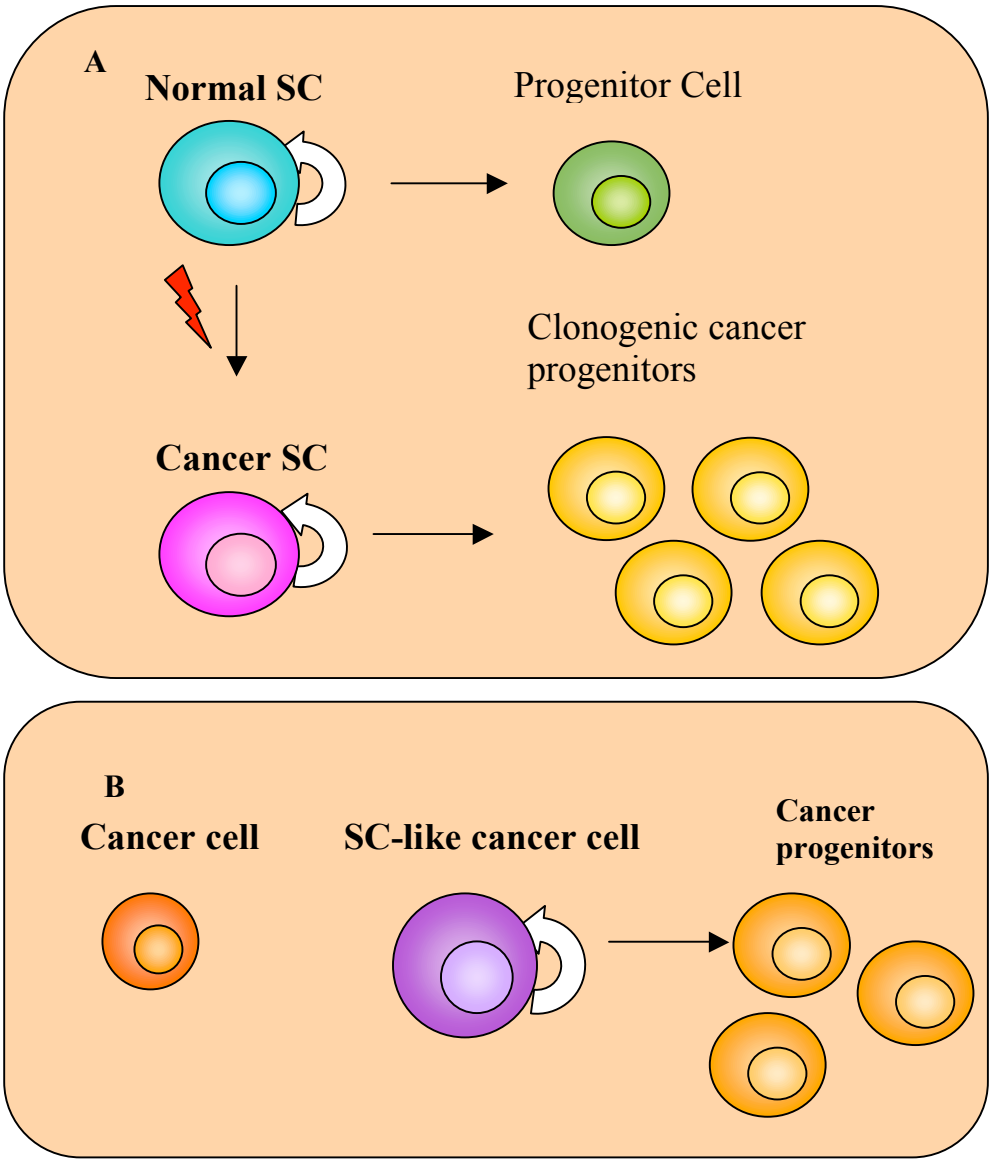


Fig. 1.1. Mechanisms of cancer stem cell formation. (A) Normal stem cells can acquire mutations resulting in transformation, or (B) transformed cancerous cells may later acquire stem cell–like characteristics.

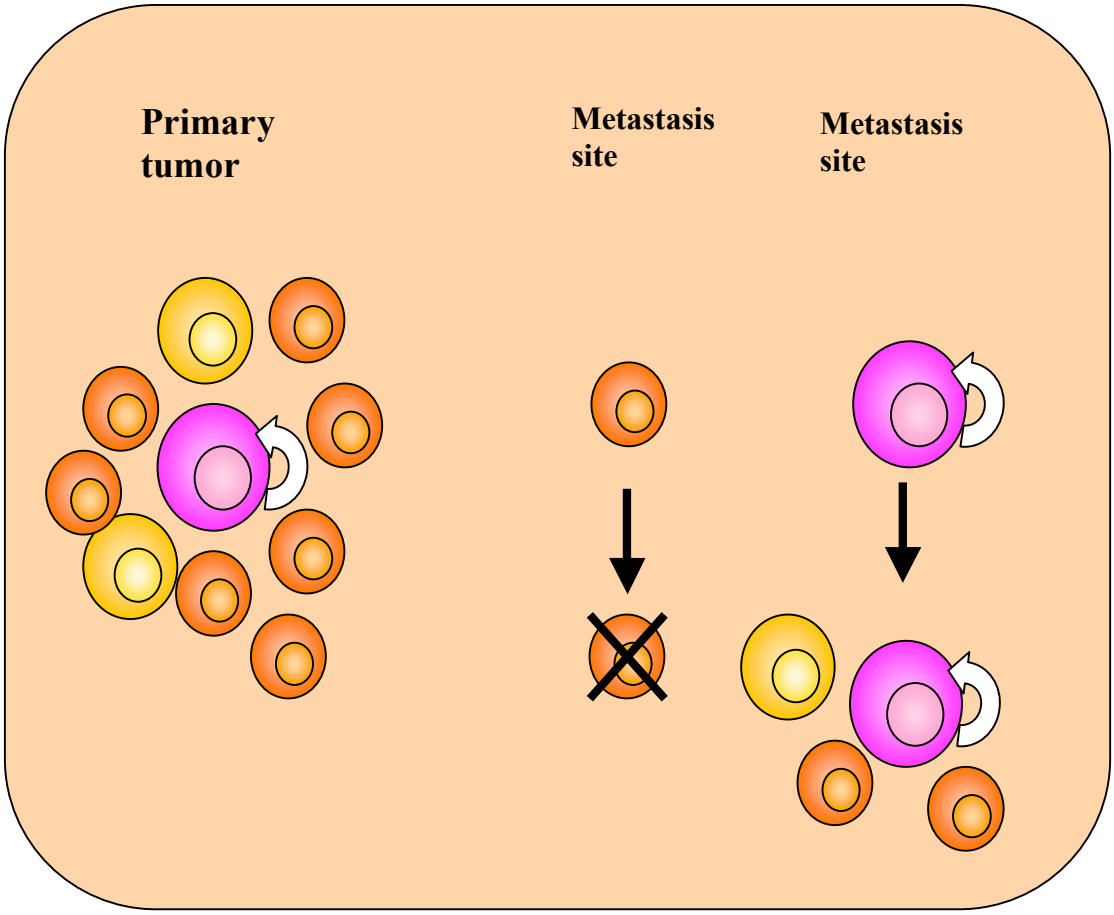


Fig.1.2. Tumor-initiating cells are cancer stem cells. Only a small population of cells in a tumor, with stem cell-like characteristics can reinitiate tumor growth or cause metastasis.

makes identification and study of cancer stem cells crucial in the treatment of metastatic neoplasia, and underlines the importance of understanding the mechanisms involved in normal stem cell proliferation and maintenance. In this work, I show that mechanisms that regulate neural stem cell proliferation in the fruit fly *Drosophila* also regulate cancer cell proliferation in advanced prostate cancer.

Development and cancer

The fundamental question of developmental biology is how a whole organism arises from a single fertilized egg. This is very like the problem of a single cancer cell escaping treatment and giving rise to new tumor. Likewise, the development of an organism requires processes like cell migration, proliferation, and angiogenesis, all of which are present in solid tumor growth and metastasis. For this reason, understanding the principles involved in developmental biology, as well as the mechanism by which cells communicate is extremely important. Several signaling molecules have been shown to coordinate development in a number of model organisms. Surprisingly, these developmental signaling pathways are evolutionarily conserved from invertebrates to humans (Echelard et al., 1993) (Kumar et al., 1996) . This has made research in the field of developmental biology in model systems especially relevant to human development and disease.

The first developmental decisions are determined by asymmetrical location of maternally positioned transcription factors and signaling molecules (Braat et al., 2004; Ephrussi and Lehmann, 1992). But as the organism becomes multicellular and begins to rely on embryonic gene products, the importance of extracellular signaling becomes apparent both in the context of cell-to-cell signaling, and in the context of long-range signaling. The major extracellular signaling pathways involved in development include the Delta-Notch pathway, the Wingless-int (Wnt)-βcatenin pathway, the Bone Morphogenetic Protein (BMP)-TGFβ pathway, the Fibroblast Growth Factor-2 (FGF-2)

pathway, and the Sonic Hedgehog pathway. These signaling pathways regulate processes such as axis formation (Akam, 1987; Marcelle et al., 1997; Zeng et al., 2001), embryonic patterning (Nusslein-Volhard and Wieschaus, 1980; Zhang and Kalderon, 2000) and cell fate (Blaess et al., 2006) in the embryo.

Some of these pathways, such as the Delta-Notch pathway, rely on direct cell-to cell communication (Artavanis-Tsakonas et al., 1999). Alternatively, the SHH and Wnt ligands can exert their effects several cell diameters away from the sending cell (Zeng et al., 2001). As sending cells secrete signaling molecules that travel through the extracellular matrix, a concentration gradient is created. Cells receiving different concentrations of ligand will adopt different fates (Figure 1.3). This suggests that different levels of signaling intensity are needed for the transcription of different genes (Lander, 2007). In fact, there are instances where the gene expression pattern, and therefore, the cell fate adopted may depend on two or more signaling pathways working together in spatial (Zhu et al., 1999) or temporal combination (Flores et al., 2000).

Cell-cell signaling regulates both proliferation and cell migration, and coordinates the (Lander, 2007)development of the organism as a whole. In the adult, communication between epithelial cells and stromal cells ensures homeostasis (Potter, 2007), and plays a role in the maintenance of stem cell niches (Palma et al., 2005; Parisi and Lin, 1998). Although much less is known about the mechanisms by which developmental signaling helps maintain a normal context in the adult epithelial tissue, a growing body of research points to dysregulation of developmental signaling pathways as a common feature of different types of cancer, including skin, pancreatic, brain, breast, and prostate carcinomas (Bale and Yu, 2001; Cronauer et al., 2003; Li et al., 2003). Alterations in developmental signaling might include abnormal activation of receptors and downstream effectors, or it can consist of overexpression of ligand that results in abnormally high levels of signaling. The latter case points to intercellular communication as a key step in the process of cancer progression, as it implies that cancer cells can abnormally signal to

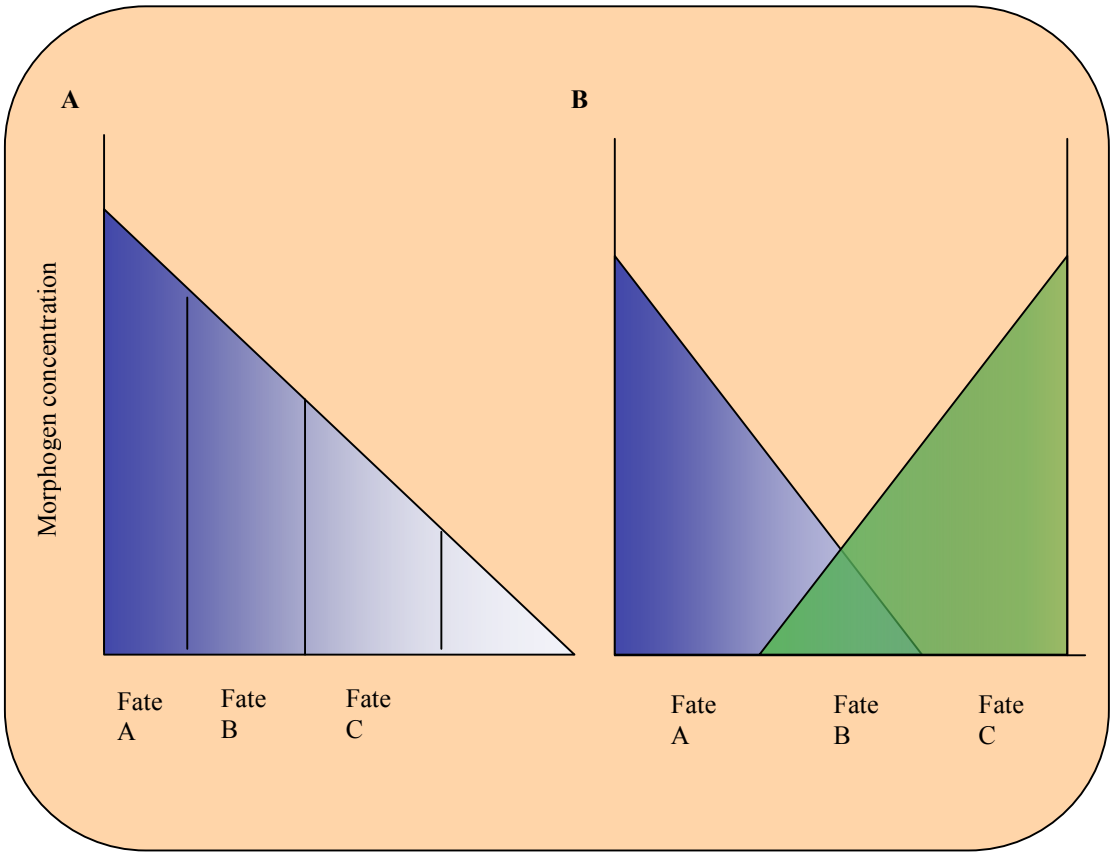


Fig.1.3. Different modes of action of morphogens. Cells interpret gradients of concentrations of (A) one or (B) more morphogens to decide cell fates.

one another and therefore escape normal regulation cues, as well as utilize the same signaling strategies used in development to aid in proliferation and metastasis. In chapter III, we show that the Sonic Hedgehog pathway plays a crucial role in proliferation of advanced prostate cancer, and that in this context, Sonic Hedgehog is regulated by the extracellular matrix proteoglycan Perlecan.

Cancer and microenvironment

Disruption of tissue microarchitecture is a defining feature of all solid tumors, and in fact the degree of disruption is often used (in addition to other characteristics) to classify the stage of the disease (Humphrey, 2007). Events that lead to rearrangements in tissue organization include loss of apical-basal polarity in epithelial cells, loss of adhesion molecules such as E-cadherin and expression of abnormal adhesion molecules, surface proteoglycans and matrix metalloproteinases (Huber et al., 2005). Taken together, these events show that in neoplasia there is a highly abnormal interaction of the tissue cells with their adjoining surroundings, and underlines the importance of understanding the normal interactions between cells and the extracellular matrix (ECM).

The ECM is a three-dimensional structure comprised of large macromolecules, including collagen, integrins, and heparan sulfate proteoglycans (HSPGs). Research performed on a variety of model systems has shown that apart from providing architectural scaffolding and contextual information (Bissell and Radisky, 2001), components of the ECM are required for facilitation of intercellular signaling by directly participating in ligand-receptor interactions (Rapraeger et al., 1991) and by aiding in the transport of long-range signaling molecules (Nybakken and Perrimon, 2002b) (Figure 1.4). During development, cells use the ECM to gather positional information through both "absolute" gradients of morphogen concentration, as well as relative concentrations of different molecules in adjacent cells (Lander, 2007) . Cells interpret information obtained through the ECM, as well as direct cell-cell interactions in order to assess their

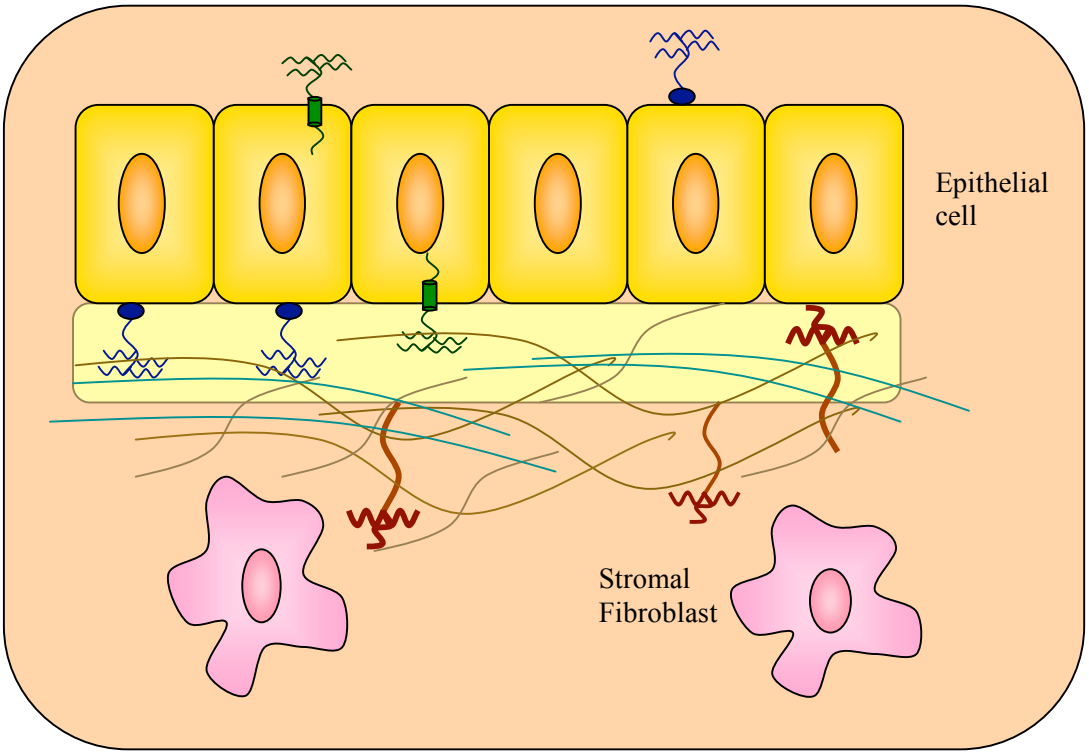


Fig.1.4. The extracellular matrix (ECM). This three-dimensional network is composed of macromolecules such as HSPG, and facilitates intercellular signaling.

tissue orientation, mediated by a system of asymmetrically localized components that provide apical-basal polarity. In adult epithelial tissues, the ECM is produced by the interaction between epithelial cells and the adjacent stroma, and is crucial to ensure appropriate cell fate and homeostasis. Alterations in ECM structure and composition can be observed in various physiological situations, as is the case of tissue repair after injury or infection. Conditions in which ECM alterations are sustained, as in chronic inflammation, often are precursors of cancerous lesions as is the case with chronic pancreatitis (Kayed et al., 2003). This leads to the hypothesis that alterations in the ECM can lead to the progression of preexisting precancerous lesions by modifying the way cells signal to one another. This has been shown to happen in case of small lung cancer (Watkins et al., 2003). It is also known that in a number of cancers such as hepatocellular, breast, and ovarian cancer, tumor cells alter the composition of surface HSPGs in a manner that allows for increased binding of growth factors ((Lai et al., 2003a) reviewed in (Fuster and Esko, 2005)).

Further study of the mechanisms by which the normal ECM helps suppress progression of tumorigenesis is needed. We can see that the ECM emerges as a new component of the cancer process, and it could be a potential target in the treatment of the disease. In this work, I show that the HSPG Perlecan, an integral component of the ECM, regulates signaling by the growth factor SHH in advanced prostate cancer. Furthermore, in Chapter IV I will describe my development of a new model of prostate cancer that allows investigation of the effect of changes in the ECM on carcinogenesis and cancer progression.

Model systems of disease

Observations in human physiology lead to hypotheses that attempt to explain the initial phenomena. However, when studying human disease most of the data available is correlative, due to the difficulty of obtaining samples, specially appropriate matching

controls. Furthermore, strong correlations, while indicative of a relationship, do not always describe a causal relationship, and there are inherent ethical concerns and environmental factors that prevent manipulation of the system. Model systems allow for a tight level of control both in terms of the environment surrounding the organism, and for precise genetic or biochemical manipulation while being relatively easy to handle and maintain. The relevance of research in model systems for human disease has been extensively shown (Brumby and Richardson, 2005). We now know that many evolutionarily distant organisms share conserved molecular and functional mechanisms. For example, the fundamental signaling pathways necessary for development are conserved from invertebrates such as *Drosophila* to humans. Emerging research on the process of aging also shows the same trend, with signaling pathways involved in aging also showing conservation across the evolutionary ladder.

Different model systems provide different advantages and disadvantages for research: *in vitro* systems such as cell lines provide the researcher with a wonderful opportunity to manipulate the system in very precise ways, while eliminating genetic background differences and environmental conditions as variables. This allows for a very clear observation of the effects of a certain treatment in terms of gene expression/ biochemical interactions, and for an accurate view of possible mechanisms. However, care must be exerted when extrapolating the conclusions drawn from in vitro systems to a whole organism due to the fact that possible interactions with other cell types, as well as the complicated processes involved in development and homeostasis may result in a different outcome *in vivo*.

Whole organism approaches to modeling disease have the advantage of providing valid results in the context of a whole organism, while still allowing the researcher to control for genetic background and environmental factors. Still, because of the evolutionary distance between model organisms and humans, results from a model organism still need to be validated with the aid of clinical data. And, as we mentioned before, the researcher

has to take into account developmental and homeostatic processes that may modify the conclusions drawn from the observed result.

From all this we can conclude that model systems are extremely useful in testing initial hypotheses based on observation of physiological data, especially in terms of causal relationships and mechanisms. The researcher can then validate the working hypothesis in the context of human disease with the aid of correlative data from clinical studies. Initial validation of results then provides a framework where the model system can be further used to test additional hypothesis, or discover additional components and/or levels of regulation that may influence the disease of interest.

The Drosophila *model system*

The fruit fly *Drosophila melanogaster* is one of the leading model systems for the study of developmental signaling pathways and their effects on cell proliferation. It has a relatively small genome, a short life cycle, and can be easily and affordably maintained in a laboratory. The extensive knowledge of *Drosophila* biology, genetics, development, and the availability of sophisticated genetic and molecular tools allow for extensive and detailed analysis of genetic and molecular interactions between signaling pathway components. *Drosophila* development has been studied extensively, and systems such as the *Drosophila* embryo and imaginal discs have been used in characterizing the regulation of proliferation throughout development by extracellular signaling pathways. More recently, *Drosophila* has become a fruitful model for stem cell studies. Most *in vitro* analyses use dividing stem cells. However, in a living organism stem cells are frequently mitotically quiescent and must be reactivated only when stem cell progeny are required. The control of stem cell proliferation in vivo requires careful control of cell cycle arrest and subsequent reactivation of proliferation by developmental cues. An ideal model to study reactivation of proliferation in stem cells is provided by the *Drosophila*

Central Nervous System (CNS), which undergoes two phases of neurogenesis separated by a period of quiescence.

Development of the Drosophila *central nervous system (CNS)*

The development of the CNS in *Drosophila* involves two phases of neurogenesis. The first phase occurs during embryogenesis, and involves the delamination of neural stem cells, or neuroblasts, from the neuroectodem following specification of neural fate by lateral inhibition (Urbach et al., 2003), a process that involves Delta-Notch signaling (Artavanis-Tsakonas and Simpson, 1991). After delamination, the neuroblast divides asymmetrically to generate another neuroblast and a ganglion mother cell; this process will continue throughout embryogenesis. At the end of embryogenesis, distinct populations of neuroblasts enter a period of quiescence, where they exit the cell cycle at the G1-S transition.

The postembryonic phase of neuroblast proliferation involves the reactivation of previously quiescent neuroblasts in the first and second instars of larval development. During the late 1st instar, the central brain and optic lobe neuroblasts resume asymmetric division, and in the early second instar, thoracic neuroblasts reactivate proliferation (Figure 1.5). This makes the postembryonic phase of neuroblast proliferation an ideal model in which to study the signaling cues that instruct stem cells to resume proliferation.

Earlier studies (Datta, 1995) had shown that the *terribly reduced optic lobes (trol)* locus is necessary for activation of proliferation in quiescent neuroblasts of the *Drosophila* larval brain. The *trol* loss-of-function mutants fail to resume normal neuroblast proliferation, resulting in significantly lower numbers of actively dividing neuroblasts. *trol* encodes the homolog of mammalian Perlecan, and was shown to exert its effect by

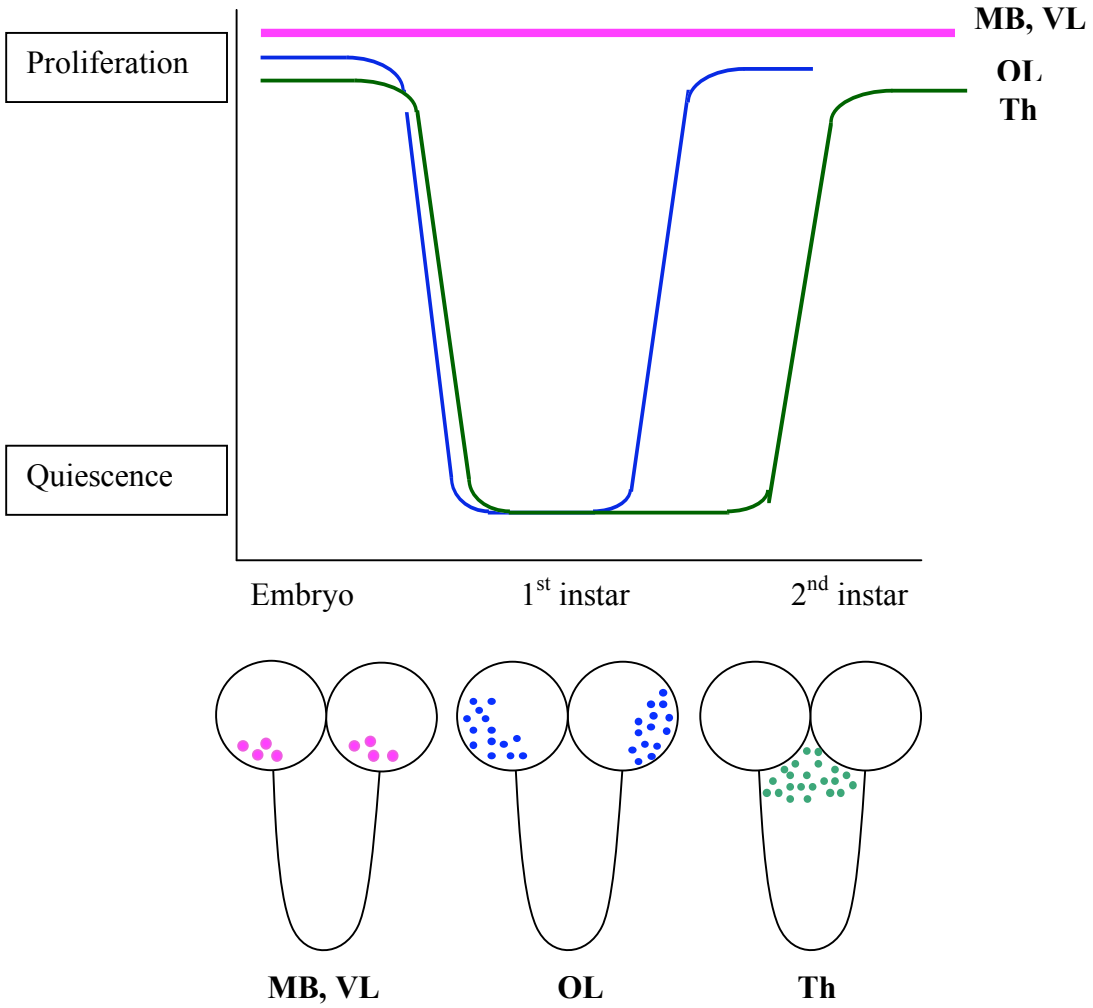


Fig.1.5. Specific subsets of neuroblasts reactivate proliferation at developmentally regulated times. Neuroblasts (NBs) in the larval brain can be divided into three main populations: The mushroom body (MB) and ventral lateral neuroblasts (VL) divide throughout embryogenesis and larval life. Optic lobe (OL) neuroblasts enter quiescence at the end of embryogenesis and reactivate proliferation at mid-first instar; Thoracic neuroblasts (Th) reactivate proliferation at late first instar.

interaction with Hedgehog (Hh), homologous of the Hh family of proteins in humans, and Branchless (Bnl), homologous to human FGF-2 (Park et al., 2003). These signaling pathways play a crucial role in development and differentiation, and are conserved from *Drosophila* to mammals.

The Hedgehog signaling pathway

The Hedgehog signaling pathway is highly conserved among invertebrates and vertebrates. It plays crucial roles in the development of segmental polarity, anteroposterior patterning of the wing, and neuroblast reactivation after quiescence in flies, as well as neural tube development and patterning of limbs in vertebrates (Lum and Beachy, 2004). Although the basic signaling process has been extensively studied and is now well understood, new pathway components and recently discovered interactions expose the need for further exploration of the unique mechanisms involved in Hh signal transduction.

In the fruit fly, Hh signal transduction results in the transcriptional activation or inhibition of Hh response genes (Figure.1.6). In the absence of Hh, the 12-transmembrane domain receptor patched (Ptc) is able to inhibit the 7-transmembrane protein Smoothened (Smo). This results in the association of Smo with the scaffolding protein Costal-2 (Cos-2), the transcription factor Cubitus interruptus (Ci), and the kinases Fused, Protein Kinase A (PKA), Glycogen synthase kinase 3 (GSK-3) and Casein Kinase1 (CK1) (Hooper and Scott, 2005). This complex, termed complex I, promotes the phosphorylation of Ci, and its subsequent processing into an N- terminal fragment termed CiR. CiR can then enter the nucleus, where it acts as a transcriptional repressor. A large fraction of the existing unprocessed Ci in cells is associated with Suppressor of Fused (Su(Fu)), which in the absence of Hh signaling retains unprocessed Ci in the cytoplasm.

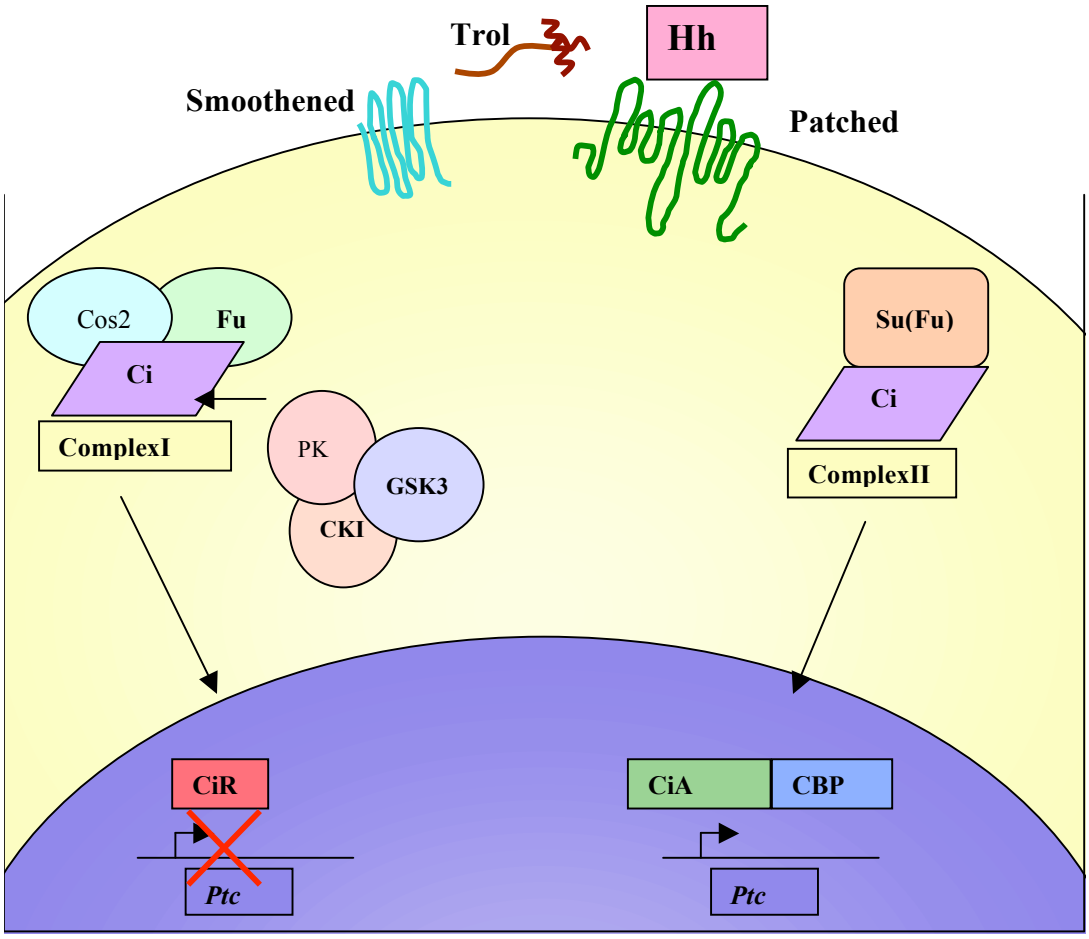


Fig.1.6. The Hedgehog signaling pathway. In the absence of the ligand Hedgehog (Hh), the receptor Patched represses smoothened. This allows for binding of the transcription factor Ci to bind complex I. This causes cleavage of Ci into a repressor form, CiR, which represses transcription. When hedgehog binds Patched, the repression on smoothened is relieved, inhibiting cleavage of Ci. Thus, Ci can enter the nucleus in its activator form, CiA.

When the Hh ligand binds to Ptc, the inhibition of Ptc on Smo is relieved. This in turn caused phosphorylation of Smo, and dissociation of complex I. Fu and Ci then associate with Su(Fu), causing release of the full length Ci (CiA), which can then enter the nucleus and function as a transcriptional activator of Hh response genes including its receptor *ptc*. Genetic interaction studies have shown that the proteoglycan Trol is requires for full-strength Hh signaling in the Drosophila CNS (Park et al., 2003), athough the mechanism of this modulation is still unclear.

This pathway is essentially conserved in vertebrates, although gene families exist for each of the *Drosophila* components; thus, in vertebrates there are three Hedgehog proteins (Sonic Hedgehog, Desert Hedgehog and Indian Hedgehog), two Ptc homologues (PTCH1 and PTCH2), and 3 Ci homologues, (GLI1, GLI2 and GLI3) with partially redundant functions.

Importance of postranslational modifications of Hh proteins

It has been shown that the Hedgehog protein acts as a morphogen (Roelink et al 1995), and that it is capable of both short range and long range signaling. This is thought to be dependent on postranslational modifications of Hh, as unmodified Hh does not have the same effects as the fully processed one (Mann and Beachy, 2004). The Hh protein is produced as a precursor, which then undergoes autocatalytic cleavage on its C-terminus. At the same time a cholesterol moiety is added. Hh is further modified by the addition of a palmitoyl group on its N-terminus (Chamoun et al, 2001) (Figure.1.7.)

Recent findings point to the existence of soluble multimeric forms of Hh and to the association of cholesterol modified Hh with lipoproteins (Panakova et al, 2005). Studies aiming to characterize the role of cholesterol and palmitoyl modifications of Hh in signaling have yielded diverse results. Early studies (Porter et al, 1995) showed that a

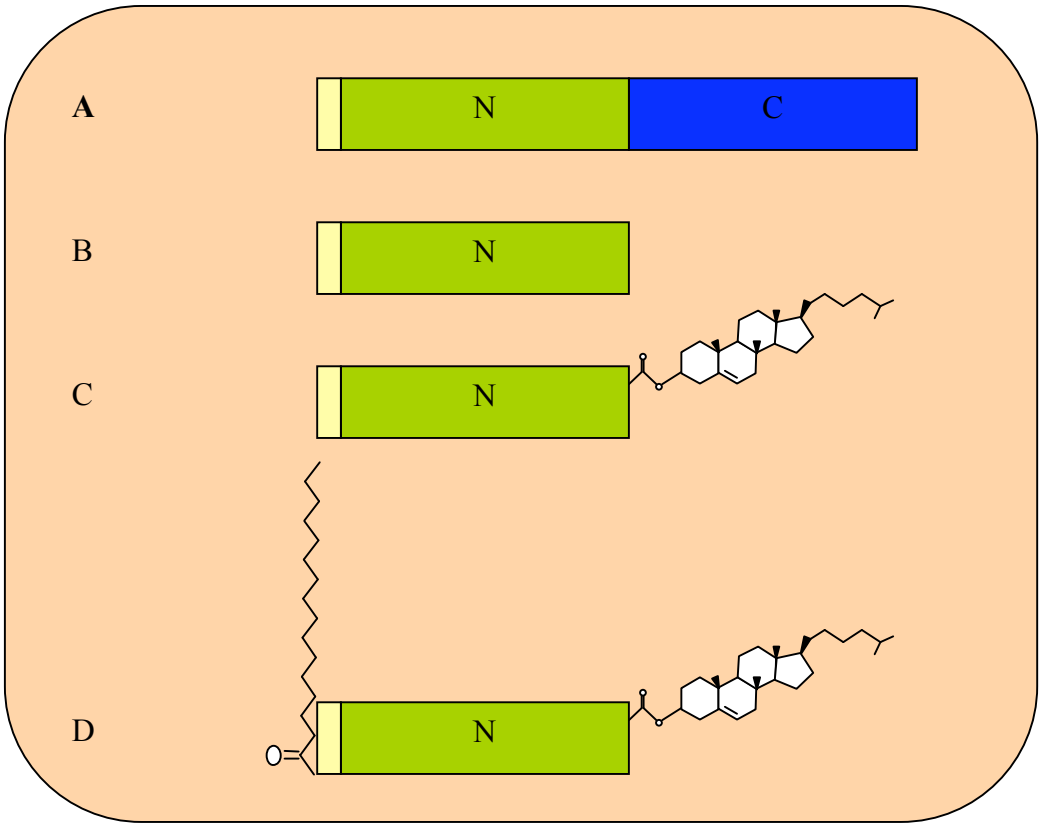


Fig.1.7. Postranslational modifications of Hedgehog proteins. Hedgehog is translated as a precursor protein (A), which undergoes autocatalytic processing mediated by its C-terminal portion C-terminus. At this time a cholesterol group is added (C). Hh is further modified by addition of a palmitate group (D). Many studies have used a truncated form of the protein without any modifications, termed HhN (B).

truncated, unmodified form of Hh, HhN, retained signaling ability in the *Drosophila* embryo. These results were emulated in vertebrates. However, further studies (Zeng et al, 2001, Chen et al, 2004) indicated that in vertebrates the processed form of Hh forms a soluble multimer that is capable of stronger signaling for a longer distance, whereas the unprocessed form remains monomeric. In the fly wing disc, cholesterol-modified Hh has differential requirements for long-range signaling, compared to unmodified HhN. Thus there are significant differences in the form and function of Hh depending on the presence or absence of the C-terminal cholesterol modification, raising the question of how well unmodified HhN mimics Hh (or ShhN mimics Shh) in experimental situations and how the effects of HhN differ from signaling by endogenous Hh. I will explore this question further in Chapter II.

It has been shown that a cholesterol-rich diet correlates with a higher risk of prostate cancer (Bravi et al., 2006); recent reports point to a causal relationship, as the use of cholesterol-lowering drugs such as statins correlates with lower risk of prostate cancer (Platz et al., 2005). Furthermore, statins induce cell cycle arrest and apoptosis in prostate cancer cell lines (Hoque et al., 2008). Since cholesterol levels regulate certain secretion processes such as the release of prostasomes (a specialized type of secretory vesicles) from prostate epithelial cells (Llorente et al., 2007), it is possible that alterations in the levels of cholesterol may be important for Shh processing, or may regulate its association with liposomes, thereby modifying its signaling range.

Heparan sulfate proteoglycans as modulators of growth factor signaling

One of the fundamental questions that remain to be solved is how Hh, a highly hydrophobic molecule, is capable of both short range and long range signaling. One of the proposed explanations involves the movement of Hh molecules along basement membranes (Gallet et al., 2003). Basal membranes are composed of a rich array of molecules that maintain the structure and shape of tissues, as well as having an important

function in cell- to-cell and long- range signaling. Among these molecules, the Heparan Sulfate Proteoglycans (HSPGs) have been identified as integral components of basement membranes in numerous tissues. These particular types of glycoproteins consist of a protein core and Heparan Sulfate chains; the three main types of HSPGs are Syndecans, Glypicans, and Perlecans (Figure 1.8). The earliest clues about HSPG function come from the studies of Fibroblast Growth Factor 2 (FGF-2) and its specific interaction with Syndecan; it is now known that Syndecan is necessary for the formation of a ternary complex with FGF-2 and the FGF receptor and elicits signal transduction (Rapraeger et al, 1991). Genetic interaction studies have highlighted the importance of HSPGs in Hh signaling (Bellaiche et al, 1998; Borneman et al, 2004; Desbordes et al, 2003). Biochemical studies have shown that heparan sulfate proteoglycans in the extracellular matrix, such as Trol, are able to bind Hh (Park et al, 2003). Murine Perlecan is also able to bind Shh, suggesting that these interactions are evolutionarily conserved.

From *Drosophila* brain stem cells to prostate cancer

The Hh signaling pathway was initially characterized in *Drosophila* (Nusslein-Volhard and Wieschaus, 1980), and homologs for the main pathway components were found to function in the same fashion in organisms ranging from zebrafish to humans. Conversely, the *Drosophila* homologs of known pathways (such as the FGF-2 homolog, *bnl*) have also been extensively studied, and found to signal in the same manner in flies and humans. Furthermore, genes involved in processes that are hallmarks of cancer, such as the process of epithelial to mesenchymal transition (EMT) have been found to cause metastasis in *Drosophila*, acting in the same coordinated fashion to control apical-basal polarity (Pagliarini and Xu, 2003). Studies in *Drosophila* to discover additional regulators of signaling pathways have revealed the existence of other Hh pathway components that include Trol, the homolog of mammalian Perlecan, and Ihog, homolog of the mammalian protein CDO. In *Drosophila*, Trol is necessary for full strength Hh signaling in larval neural stem cells (Park et al., 2003). Thus, neural stem cell

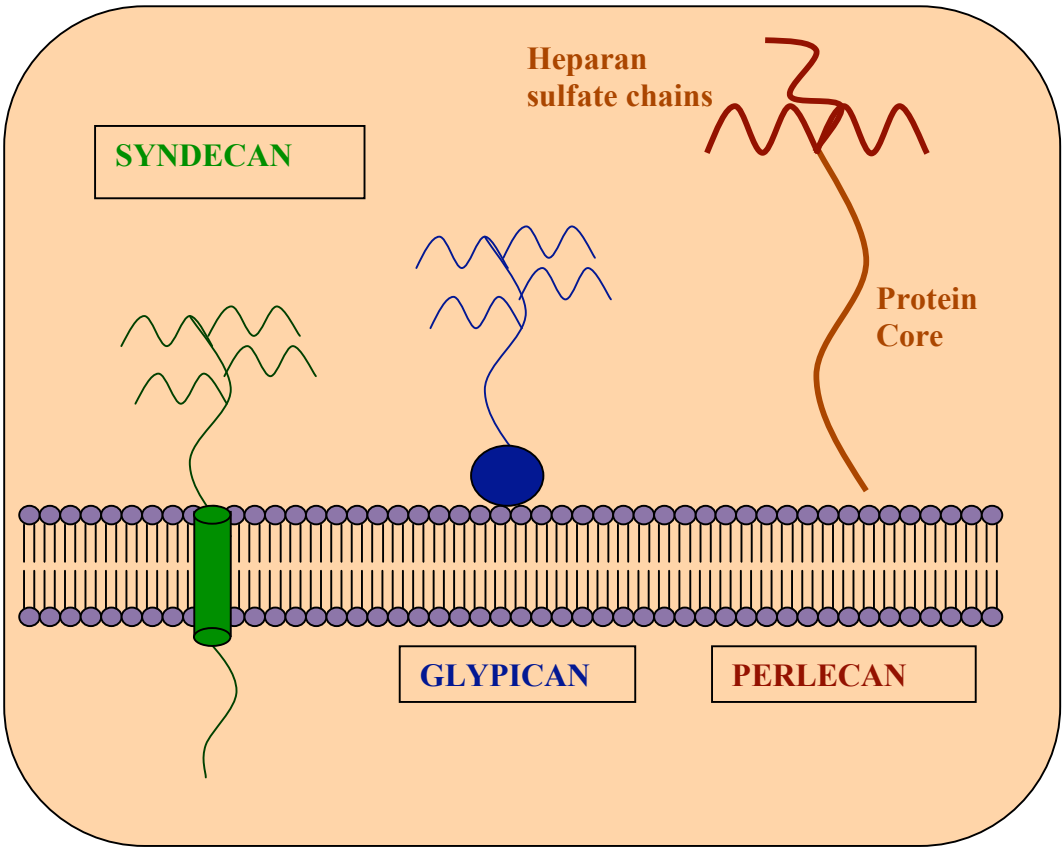


Fig.1.8. The main classes of heparan sulfate proteoglycans. HSPGs consist of a protein core with attached heparan sulfate chains. (A) Syndecans are attached to the cell surface by a transmembrane domain.(B) Glypicans are covalently attached by a glycosyl-phosphatidylinositol link. (C) Perlecan can be secreted to the exterior or remain cell-surface associated.

proliferation in the *Drosophila* larval brain depends partly on a signaling module constituted by the Hh pathway regulated by Trol. Interestingly, the mammalian homolog of *trol*, *PERLECAN*, is upregulated in a prostate cancer cell line (Iozzo et al., 1994), and the single human *PERLECAN* gene has been mapped to 1.p.36. This corresponds to the CABP locus, a locus linked to increased risk of both prostate cancer and Glioblastoma multiforme, a brain cancer of astrocytic origin (Gibbbs et al, 1999).Human genetic studies have thus provided initial correlative data about the possible involvement of Perlecan in both brain and prostate cancer, and earlier studies in *Drosophila* show that Perlecan is needed for Hh signaling to proceed properly in neural stem cells. This raises the question of whether the signaling module functioning in the *Drosophila* larval CNS might be also functioning in prostate cancer. I therefore hypothesize that Perlecan is the CABP locus gene, and that it regulates SHH signaling in prostate cancer. This hypothesis is addressed in Chapter III.

Clinical relevance of prostate cancer

Prostate cancer is the second leading cause of death from cancer, and the most commonly diagnosed form of cancer in men. According to the American Cancer Society, prostate cancer has accounted for about 10% of deaths in US males in 2007 with 218,000 new cases and 27,000 deaths in 2007 alone (SEER registry Database 2008). Old age is the major risk factor for prostate cancer—as the graying of America increases, we expect prostate cancer incidence to increase. Men have a 1 in 6 possibility of developing PCa (ACS, 2007). Initial treatment consists of prostatectomy or radiation, with a fairly good prognosis if the tumor is detected at an early stage, and has a slow growth. However, many tumors are detected at later stages, and recurrence of tumors after surgery may be observed (Feldman and Feldman, 2001). Initially, tumor growth is androgen-dependent, which makes androgen–ablation therapy the treatment of choice, yet the median length of response to the treatment is 10-24 months (So et al, 2005). Tumors progress from androgen dependence to androgen independence. Androgen-

Independent Prostate Cancer (AIPC) is lethal, invasive and metastatic, and no current treatments can help extend lifespan. A better understanding of the mechanisms underlying the onset of advanced PCa is needed in order to develop better diagnostic tools and new treatment strategies.

Age as a risk factor for prostate cancer

Studies show that two out of three prostate cancers are diagnosed in men over 65 years of age. The most recent statistics from the SEER registry show that the peak incidence of prostate cancer happens in men 70-74 years old. Not all cancers share this trend: for example the peak incidence of testicular cancer happens at a much younger age according to the Surveillance epidemiology and End results (SEER) registry of the National Cancer Institute. It has been thought that the reason most cancers reach their peak prevalence in old ages is because it takes a long time for cells to accumulate the set of mutations needed for the cancerous lesion to form. While the latter is true, little consideration has been given to the fact that the actual aging process may alter conditions that influence the behavior of a preexisting cancer lesion. When we couple this to the emerging paradigms on cancer research, we can see that there is a clear trend towards evaluation of the impact of aging in the signaling and microenvironmental changes that might affect the outcome of an initial precancerous lesion.

For a long time, aging has been thought of as a progressive decline of the systems that have to function to sustain life. However, it is becoming clear that aging is a tightly controlled process, and that there are a number of signaling pathways that affect both the onset and the rate of aging (Kenyon, 2001). The fact that aging is a complex biological process, akin to development or behavior in which many regulating systems cooperate to produce results, has obscured and complicated the definition and study of aging. But in the last two decades, our understanding of the aging process has been greatly increased, and this has led to emerging paradigms and approaches to the further understanding of

the mechanisms underlying aging. These approaches focus on the study of mutations that increase lifespan (the best characterized measure of aging) in model organisms, as well as studying age-related diseases in humans.

Researchers have approached the study of aging by studying syndromes like Hutchinson-Gilford progeria or Werner syndrome, which are characterized by accelerated aging. These have revealed mutations in the WRN gene, which encodes a recQ helicase, or the LMNA gene, which encodes lamin A (Kipling et al., 2004). These discoveries highlight an important role of maintenance of DNA and nuclear structure in tissue homeostasis and aging. But it is likely that these proteins represent the endpoint of more complex mechanisms that may be in place to regulate the onset and development of aging. For this reason, model systems are being used to elucidate the mechanisms that regulate aging and result in destabilization of genome integrity.

For a long time, it has been known that there are a variety of means to manipulate lifespan in different organisms. The most studied of these is calorie restriction, which increases lifespan in yeast, invertebrates, and mammals (Haigis and Guarente, 2006). Physiological responses to calorie restriction include a slower metabolism, increases in stress resistance, and a decrease in reproduction. Research on the molecular basis for the effects of calorie restriction has uncovered the importance of sirtuins (a family of NAD-dependent histone deacetylases), IGF signaling, and Foxo transcription factors as important regulators of lifespan.

Initial investigations using yeast revealed that mutations in the SIR complex result in an increase of replicative lifespan, and showed that Sir2 (the first sirtuin described) has a significant impact in lifespan (Haigis and Guarente, 2006). The requirement of NAD for proper Sir2 function suggests a link between metabolism and Sir2 activity, as does the fact that calorie restriction cannot rescue the short lifespan of Sir2 mutants. Sir2

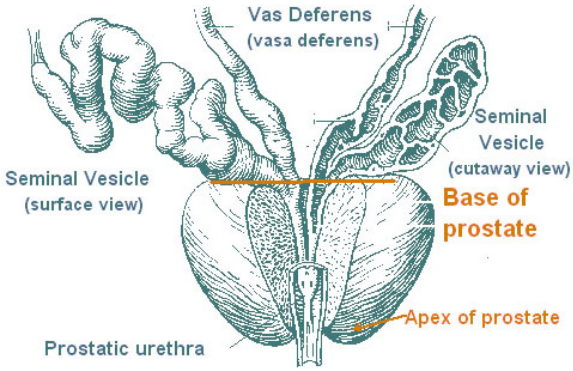
homologs have been shown to regulate lifespan in *C.elegans*, and *Drosophila*. Further investigation on the role of Sirtuins in vertebrate and mammalian aging is still needed.

Perhaps the most significant discoveries on regulation of lifespan have been discovered using the nematode *C.elegans*. In this system, calorie restriction induces a diapause state, characterized by lower metabolic rate and developmental and reproductive arrest, which enables the nematodes to survive for long periods of time until adequate food supplies become available. Studies on diapause mutants uncovered a role for the Insulin/Insulin Growth Factor (IIS) signaling pathway in restoring the animals to a full reproductive state. Further studies showed that weak mutations in the IIS pathway produced strains that retained their normal metabolism and reproductive potential while conferring an longer lifespan, thus uncovering an important role of IIS in lifespan regulation. In *C.elegans*, IIS acts by downregulating the FOXO transcription factor DAF-16 (Berman and Kenyon, 2006). DAF-16 homologs in yeast and *Drosophila* also play a significant role in regulating lifespan in these systems. From these studies, a paradigm emerges where aging is a tightly regulated process that may influence the progression of other events in the organism. While the links between aging and cancer are still the focus of much investigation and controversy, it has been shown that age affects the interactions between cancer cells and their host tissue. Reports that senescence, a cellular condition associated with aging has a significant impact on cancer cell proliferation (Krtolica et al., 2001) also suggest that these two processes may interact and affect each other.

The prostate: brief anatomical and physiological overview

The prostate is a male accessory reproductive gland that produces a complex proteolytic solution aiding the survival of sperm. It is located directly beneath the bladder, and completely surrounds the urethra. The prostate predominantly consists of acini that empty into ductules streaming into the ejaculatory ducts, which enter the base of the gland and extend anteriorly to the urethra (Figure 1.9). The prostate is subdivided into 3

A



B

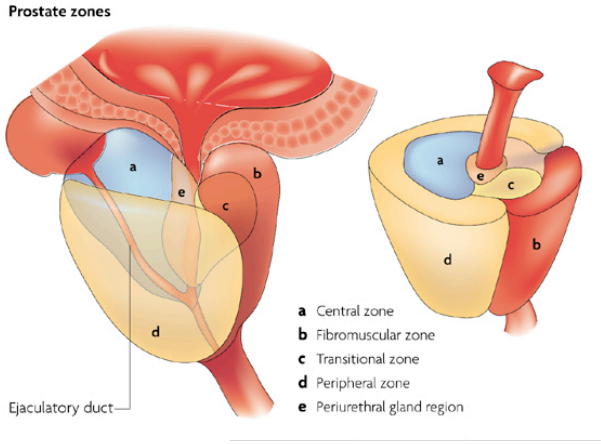


Fig.1.9. The prostate. (A) location of the prostate in the male reproductive system. (B) The prostate zones. Modified from (De Marzo et al., 2007), and (SEER Registry Database, 2008)

distinctive zones: peripheral, central, and transitional. Seventy percent of all prostatic carcinomas arise in the peripheral zone (Abel, 2001). The prostate epithelium is composed of epithelialal, luminal secretory, and neuroendocrine cells. The stroma is composed of fibroblasts, smooth muscle cells, endothelial and dendritic cells, and infiltrating cells from the immune system (Feldman and Feldman, 2001).

Normal prostate development

Development of the prostate begins approximately at the seventh week of gestation, and is heavily dependant on androgen signaling. At this time, the male and female reproductive tracts are identical, and testosterone signaling from the male testes directs differentiation of the early prostate and male urogenital tract. At the end of the third trimester, the gland enters a developmentally quiescent state. During puberty, the prostate experiences another growth period, and the full secretory phenotype is established. Later in life, as testosterone levels drop again the prostate undergoes involution and atrophy; it is at this stage where deregulation of known processes may play a role in bringing about the onset of prostate cancer.

While androgen signaling in prostate development has been well characterized, there are a number of additional signaling pathways that are critical for prostate development. The requirement for Fibroblast Growth Factor (FGF), Bone Morphogenetic Protein (BMP), Sonic Hedgehog (Shh), and Wingless-int (Wnt) signaling in prostate development has been demonstrated **(**Yardy and Brewster 2005, Settle et al 2001, Kwabi-Addo et al 2004). For example, Shh signaling is necessary for ductal branching morphogenesis and epithelial differentiation of the prostate, although there are conflicting reports about its requirement in prostatic growth induction from the early urogenital tract (Berman et al 2004, Freestone et al 2003). It is thought that complex interplay between Shh signaling and other signals such as FGF and BMP direct branching and differentiation of the prostate in a carefully controlled manner.

Prostate cancer models

In order to gain insight into the mechanisms underlying prostate cancer onset, there have been a number of model systems developed for this purpose. Prostate and prostate cancer cell lines, derived from a variety of sources, provide a system in which genetic homogeneity is assured, and environmental conditions can be carefully controlled. These cell lines have been extensively used to identify cellular characteristics that correlate with androgen dependence/independence, invasiveness, and metastatic potential. They have also been used to study the effects of various signaling molecules and their inhibitors on cell growth and proliferation. Nevertheless, one has to consider that this is an inherently artificial system, and *in vivo* responses may be different. Other prostate cancer models include the Lobund-Wistar rat, a specific strain with an inherent predisposition to develop spontaneous metastatic, hormone-influenced prostate tumors. The Lobund-Wistar rat model has been useful to study *in vivo* tumorigenesis at the levels of spontaneous and induced tumors, and to identify genes that confer susceptibility to prostate cancer (Pollard, 1998). However, the rat model system is costly and time consuming, spontaneous tumors take years (~25% incidence at 25 months)(Pugh et al., 1994) to develop, and specific molecular interventions cannot be easily manipulated. Mouse models have used different approaches; the development of transgenic mouse models that incorporate tissue–specific control of the genome has allowed the study of the effects of different genes *in vivo* (Jin et al, 2003, Jin et al, 2003**a)**. Another strategy used a prostate-specific promoter and the SV40 inactivators of the tumor suppressors p53 and RB (retinoblastoma) to create the Transgenic Adenocarcinoma of the Mouse Prostate (TRAMP) mouse. TRAMP mice develop spontaneous invasive and metastatic prostate cancers that are heterogeneous, and have been extremely useful in screening for therapeutic chemicals (Burman et al, 2004). However, the cost and time involved in making transgenic mice, and the pleiotropic effects of many mutations limit its potential uses. A model organism complex enough to allow investigation on the effects and

mechanisms of signaling pathway activation on the onset of prostate cancer *in vivo*, but without the problems of genetic redundancy, long developmental times and inherent complexity that higher vertebrates have will be a highly useful tool in the elucidation of cancer progression mechanisms.

Developing an improved prostate cancer model in *Drosophila*

As age is the single most important risk factor in prostate cancer, incorporating changes in signaling and proliferation as the organism ages will provide crucial insight on the mechanisms of disease. Obtaining clinical correlative data proves to be even more difficult in this instance, because the subjects have to be monitored during an extremely long periods of time (as is the case with cohort studies), and initially appropriate controls may become unusable as individuals age. The main concern in developing mammalian models for aging is precisely the fact that it takes long periods of time to obtain results from aging organisms. In this instance, invertebrate organisms are very well suited for studies on aging, because they allow the researcher to obtain results in a reasonable period of time. For example, initial discoveries on the impact of the IGF pathway on aging were initially characterized in the nematode *Caenorhabditis elegans* (Kenyon, 2001). *Drosophila* has also been used extensively as an aging model, and research in this organism has provided insights into aspects of aging such as regulation of lifespan by IGF signaling and sirtuins as well as further demonstrating the evolutionary conservation of known aging pathways (Helfand and Rogina, 2003). *Drosophila* is also emerging as a useful model for the study of cancer, since it provides information on genes affecting the behaviors observed in cancer cells while providing an organismal context: four of the six hallmarks of cancer can be modeled in the fly (the exceptions are telomerase activity and angiogenesis).

As we have said above, a useful approach to understanding prostate cancer transition to metastasis will take into account emerging paradigms in cancer biology, such as the

contribution of developmental signaling pathways and the concept of HSPGs as integral parts of the microenvironment that play a crucial role in cancer progression. It will also incorporate the effects of aging in the processes mentioned above. However, since the possibility of pleiotropic effects is still a concern, utilizing a simple model in which precise genetic changes can be easily monitored will greatly help in clarifying the observations obtained. To this end, we must reconcile the fact that most simple model organisms do not have a classic prostate organ; however, even if evolutionary homology does not exist, the functional, developmental and molecular correlations that will make the organism usable for testing initial hypotheses about prostate aging and cancer may still be present. I will explore the functional and molecular correlations between the human prostate and the *Drosophila* prostate analog in chapter IV.

In summary, our understanding of complex human diseases such as prostate cancer have benefited greatly from the insights provided by model systems. I hypothesize that the molecular signaling mechanisms uncovered in studies of the model system *Drosophila* are conserved in prostate cancer and are critical for prostate cancer progression. In addition, the *Drosophila* brain model can be utilized to study the importance of cholesterol modifications that affect Hh signaling in development, and may also affect SHH signaling in prostate cancer. Finally, the development of a prostate model in *Drosophila*, a short-lived, simple organism will be useful for studying specific aspects of signaling and aging that are relevant to prostate cancer onset and progression.

CHAPTER II
INHIBITION OF Hh SIGNALING AND ENDOGENOUS *hh* EXPRESSION IN THE *Drosophila* NERVOUS SYSTEM BY HhN

Introduction

Emerging research highlights the significance of developmental signaling in cancer progression across many different types of cancer (Cronauer et al., 2003; Ruiz i Altaba et al., 2004; Wakefield and Roberts, 2002). These findings emphasize the need to examine more closely the mechanisms that affect signaling intensity, strength and range in normal and deregulated conditions. One important process that affects signaling strength is ligand processing and modification. In signaling pathways such as Sonic Hedgehog (SHH) and Wnt, protein ligands may be modified through a series of lipid modifications that affect signaling intensity and ligand transport. In this context, the importance of cholesterol modifications in SHH signaling has been extensively described (Gallet et al., 2006; Nybakken and Perrimon, 2002a; Porter et al., 1996b)

It is well-known that the risk for prostate cancer increases with a diet rich in saturated fat and cholesterol. Recent reports have shown a positive correlation between high cholesterol levels (hypercholesterolemia) and prostate cancer (Bravi et al., 2006). Cholesterol has also has been shown to augment cell survival in prostate cancer models (Zhuang et al, 2005). Since cholesterol levels can influence the rlelase of specialized secretory vesicles from prostate epithelial cells (Llorente et al., 2007), it is possible that nutritionally supplied levels of cholesterol may impact the formation of cholesterol-modified SHH ligand, and thus have an effect on signaling strength and range, and an impact on cell survival.

Cholesterol modification is an important step in the processing of mature SHH ligand (Zeng et al., 2001). SHH is initially translated as a precursor protein. It undergoes and autocatalytic processing reaction involving internal cleavage between conserved Gly-Cys residues. The reaction involves two secuantial nucleophilic displacements; the firstone replaces the Gly-Cys peptide bond with a thioester bond; the second displaces the sulphur and severs the link between the N and C- termini of the protein. During this second displacement, a cholesterol moiety is added (Mann and Beachy, 2000). The autoprocessing reaction is mediated by the carboxy-terminal domain of the precursor protein, which has no additional function. After cleavage, SHH undergoes further modification with the addition of a palmitoyl group (Pepinsky et al., 1998). The role of lipid modifications in Hh signaling was the subject of much controversy; initial studies suggested that the unmodified N-terminus of the protein (HhN) retained full signaling ability. However, this interpretation was challenged with various studies that reported the importance of C-terminal cholesterol modification in the strength and range of signaling (Cooper et al., 2003; Gallet et al., 2006; Zeng et al., 2001). Currently, it is thought that cholesterol modification plays a fundamental role in the assembly of Hh multimers and their transport through tissues. The manner of Hh transport has also been a subject of extensive study, given the apparently contradictory notion of a cholesterol-modified molecule that has to travel though many cell diameters to exert some of its functions. Some of the proposed mechanisms for this long-range transport include cytonemes, soluble multimers, lipoproteins, and transport through Heparan Sulfate Proteoglycans (HSPGs).(Eaton, 2006; Han et al., 2004; Panakova et al., 2005)

The Hedgehog (Hh) pathway was initially characterized in the model organism *Drosophila*, where it plays an important role in developmental processes, such as embryo and imaginal disc patterning (Porter et al., 1996a) (Tabata and Kornberg, 1994) (Capdevila and Johnson, 2000). Hh also plays a role in stem cell proliferation in various tissues (Parisi and Lin, 1998; Park et al., 2003). The requirements for proper Hh function have been shown to be context dependent: for example, the proteoglycans involved in

Hh signaling in the *Drosophila* wing disc are the Glypicans Dally and Dally-like (Desbordes and Sanson, 2003; Han et al., 2004; Lum et al., 2003), while the proteoglycan modulating Hh signaling in the *Drosophila* larval brain and Sonic Hedgehog signaling in human prostate cancer is Trol/Perlecan (Datta, 1995) (Datta, 1999; Park et al., 2003). Since many aspects of Hh signaling have proved to be tissue-specific, I set out to examine the contribution of cholesterol modifications to Hh signaling in the context of neural stem cell proliferation in the *Drosophila* larval brain. In our system, unmodified Hh (HhN) had a dominant negative effect in neural stem cell proliferation. This effect was shown to be dose-dependent (Datta, 2007). To further examine the mechanisms involved in HhN inhibition of neural stem cell proliferation, I evaluated the levels of endogenous *hh* signaling in HhN overexpressing larvae. My results show that levels of both Hh signaling and endogenous *hh* expression are downregulated when HhN is overexpressed. This raises the possibility that a negative feedback loop is in place to accurately monitor and maintain the levels of Hh signaling.

Materials and methods

Fly stocks and genetics

The stocks *w ; UAS-hh* and *w ; UAS-HhN* were obtained from Dr. Phil Beachy. The homozygous viable *y w ; hs-GAL4* line was a kind gift of Dr. Bruce Baker.

Developmental staging

Developmental synchronization was carried out as previously described (Caldwell and Datta, 1998; Datta, 1995; Park et al., 2001; Park et al., 2003). Flies were allowed to lay eggs on apple juice agar plates with fresh yeast overnight or for about 24 hours. For staging of synchronized first instar larvae, the plate was first cleared of any larvae and newly hatched larvae were collected in one hour windows and placed on new apple juice plates with yeast at the experimental temperature (25°C unless otherwise specified).

Quantitative real-time PCR

Whole first instar brains were used for RNA isolation. Total RNA was isolated using the RNeasy RNA isolation kit (Qiagen) following manufacturer's directions. Samples were DNAsed and reverse transcribed using oligo dT primers and the Taqman Reverse transcription kit (Applied Biosystems). cDNA was used to perform quantitative Real Time PCR in a ABI PRYSM 7700 cycler using SYBR Green Master Mix (Applied biosystems). All qRT-PCR reactions were carried out in triplicate at three different template concentrations to ensure that we were within linear template range. Primer sequences are available upon request. Expresssion of *ß-actin* was used as an internal control. Data were analyzed using the delta-delta calculation method to yield fold change compared to controls.

Results

Our previous data that HhN inhibits neuroblast proliferation in a dose-dependent manner (Datta, 2007, (Figure 2.1A) raised the possibility that HhN decreased neuroblast proliferation by inhibiting endogenous Hh signaling. To test this hypothesis, I sought to evaluate the levels of Hh signaling in first instar larval brains overexpressing HhN and in control larvae. Figure 2.1B shows that expression levels of the response gene *ptc* are significantly decreased in HhN overexpressing larval brains in comparison with wild type brains. I also wanted to monitor the expression of the endogenous *hh* gene. Since the *HhN* transgene was constructed using a truncated *hh* cDNA, I was able to design primers that would amplify only the endogenous *hh* message. These primers are located at positions 1481 and 1538 of the *hh* gene, well after the natural *hh* cleavege site (Figure 2.1B). Interestingly, levels of endogenous *hh* expression were also significantly decreased, suggesting that the decrease in Hh signaling was at least

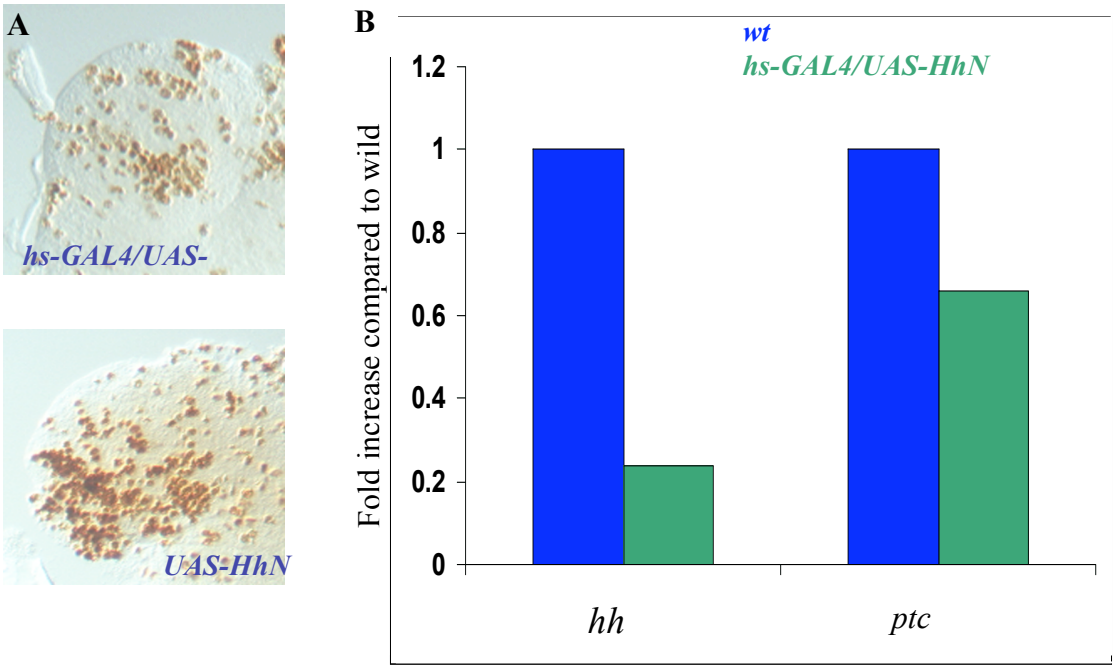


Fig.2.1.HhN overexpression decreases Hh signaling and endogenous *hh* expression**.** **(**A) HhN overexpression phenotype: Immunohistochemistry against BrdU shows fewer BrdU-incorporating cells in HhN overexpression brains. (B) qPCR showing endogenous *hh* and *ptc* expression in HhN overexpression brains vs wild–type.

partially due to a decrease in the levels of endogenous Hh ligand. This is consistent with the possibility that a negative feedback loop is in place to regulate levels of Hh signaling in the *Drosophila* larval brain.

Discussion

Previous studies (Datta, 2007) showed that HhN acts as an inhibitor of neuroblast proliferation in a dose-dependent manner in the first instar larval brain. Since Hh is an activator of neuroblast proliferation, this would suggest that HhN overexpression causes a decrease in the amount of active Hh signaling in the brain. However, this result does not address how the decrease of Hh signaling in the brain affects the specific cells implicated in neuroblast proliferation. Further elucidation of this mechanism is needed, yet further exploration of the HhN phenotype in the brain has been impaired by the instability of the *GAL4* driver used to overexpress *HhN* in the first instar larval brain (Barrett and Datta, 2007). The decrease in neuroblast proliferation is a departure from common HhN overexpression phenotypes, which evidence lower levels/reduced range of Hh signaling but never show the opposite phenotype to cholesterol modified Hh expression. Given that the range and activity of the Hh ligand is regulated by HSPGs, I hypothesized that HhN might be acting as a competitive inhibitor of cholesterol-modified Hh for HSPG modulation in the larval brain. To test this hypothesis, I first set out to evaluate the levels of Hh signaling when HhN is overexpressed. My results show that when HhN is overexpressed, there is a significant decrease in the levels of expression of the response gene *ptc*, indicating decreased Hh signaling activity. Given that our qPCR technique allowed me to differentiate between transgenic and endogenous *hh* expression, I was able to specifically evaluate levels of endogenous *hh* expression. My results showed that HhN overexpression also results in the decrease of endogenous *hh* message levels. Together with the decrease in *ptc* expression, and the decreased neuroblast proliferation phenotype, these results suggest a negative feedback loop in Hh signaling, where *hh* is expressed in accordance to the levels of Hh signaling present. My

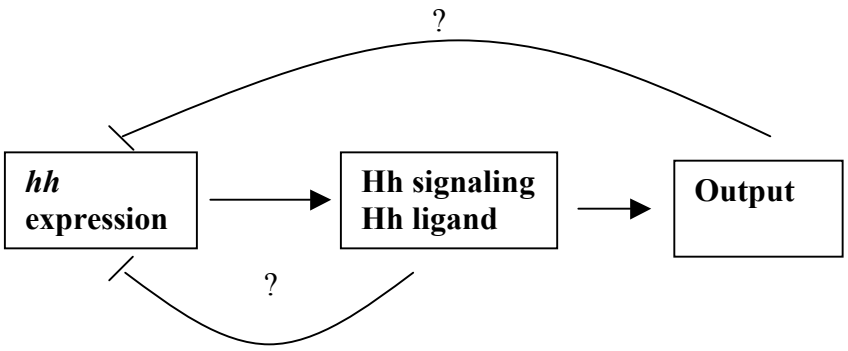


Fig.2.2. *hedgehog* expression is controlled by a negative feedback loop in the *Drosophila* larval brain. Mechanisms underlying this feedback loop are still under study.

studies imply the presence of negative regulation of *hh* expression by Hh signaling (Fig. 2.2), a novel mechanism of regulation for the Hh pathway.

Extensive investigation in the field of Hh signaling has shown differences between the activity of unmodified and cholesterol-modified Hh signaling, where the unmodified form has lower activity levels than the cholesterol-modified form (Dawber et al., 2005; Su et al., 2007). However, most of these studies were executed in genetic backgrounds where there was some contribution to Hh signaling by the endogenous gene. This raises the possibility that the observed phenotype of lower signaling was not due to decreased activity of the hhN ligand, but due to a decrease in the levels of endogenous Hh signaling caused by hhN. This would imply that HhN has little to no signaling activity. Clonal analysis with flip-out *hh* null clones overexpressing HhN (Gallet et al., 2006) suggest HhN is able to signal, but at lesser intensity than cholesterol- modified Hh in the wing disc. The difference in signaling intensity have been attributed to the inability of unmodified Hh to multimerize, or associate with HSPGs for proper transport and gradient formation (Porter et al., 1996b) (Guerrero and Chiang, 2007; Su et al., 2007; Wendler et al., 2006). These studies rely on differential response gene expression to assay levels of Hh signaling activity. Alternatively, transgenic expression of tagged Hh is followed through tissues such as the wing disc to evaluate transport. These approaches cannot differentiate between exogenous and endogenous Hh signaling since target gene activation cannot be used to distinguish between transgenic and endogenous levels of signaling. Similarly, evaluation of Hh signaling with the use of tagged Hh ligand does not take into account the contribution of the endogenous Hh present in the tissues. Furthermore, strong Hh (or HhN) overexpression could mask the negative feedback phenotype. In this context, HhN overexpression phenotypes would depend on the endogenous Hh backgound, as well as on HhN over expression. This raises the possibility that part of the HhN phenotype observed in some of these studies may actually be caused by the decrease in endogenous *hh* signaling.

My preliminary results suggest endogenous *hh* expression is regulated by the overall amount of Hh ligand present. These issues raise the possibility that in the larval brain, overexpression of an unmodified Hh form with less signaling efficiency, coupled with downregulation of endogenous Hh signaling, results in a strong decrease of Hh signaling. The resulting low levels of Hh signaling cannot sustain activation of neuroblast proliferation. The differential response to HhN in *Drosophila* neural stem cells compared to other systems such as the wing disc allowed us to uncover a potential novel mechanism of Hh regulation. This emphasizes the need to use different developmental systems in order to obtain an integrated picture of signaling function and mechanisms. If HhN overexpression causes a decrease in neuroblast proliferation by negatively regulating levels of endogenous Hh ligand and Hh signaling, HhN could be used as a therapeutic agent to decrease abnormal Hh signaling in specific tissues. Future directions aim to elucidate the mechanisms underlying the negative regulation of *hh* expression by Hh signaling, and the nature of the Hh/HhN competition. Possible mechanisms include differences in the assembly of Hh multimers, association of Hh with HSPGs for correct transportation and gradient formation through the affected tissues, or binding of Hh to components required for detection of Hh signaling particles by responding cells.

CHAPTER III
PERLECAN INHIBITION OF SHH- DEPENDENT PROLIFERATION IN PROSTATE CANCER*

Prostate cancer is the most commonly diagnosed cancer in men. According to the ACS, there were more than 27,000 deaths from prostate cancer in 2007 alone. Most men that die of prostate cancer succumb to Advanced Prostate Cancer, a highly aggressive and metastatic form of the disease that is refractory to androgen ablation therapy, the most common treatment for recurrent prostate cancer. A need to develop treatment strategies that focus on the metastatic aspect of the disease highlights the need to understand the mechanisms that underlie prostate cancer progression.

The emerging paradigms of cancer biology focus on the need to integrate our knowledge of many complex biological processes in our understanding of prostate cancer. A growing body of research points to abnormal regulation of developmental signaling pathways as a major factor in cancer progression (Feng et al., 1997; Li et al., 2003; Ruiz i Altaba et al., 2004; Sancho et al., 2004; Wakefield and Roberts, 2002). One of these pathways is the Sonic Hedgehog pathway (SHH), which has been implicated in both prostate development and numerous types of cancer. SHH signaling is conserved through evolution, allowing study of this pathway in model systems such as the fruit fly *Drosophila*. For example, our studies in the *Drosophila* CNS have identified the proteoglycan Trol, homolog of human PERLECAN, as a major regulator of the Hedgehog (Hh) pathway in neural stem cells.

---

* Portions of this chapter reprinted with permission from Sanchez, P., Hernandez A. M.,Stecca B.,Kahler,A.J., DeGueme,A.M., Barret,A., Beyna, M., Datta,M.W., Datta,S., and Ruiz I Altaba A. (2004). Inhibition of prostate cancer proliferation by interference with SONIC HEDGEHOG-GLI1 signaling. Proc.Natl.Acad.Sci USA 101 (34), 12561-12566.

Parallel studies in human genetics highlight potential loci linked to increased risk of prostate cancer; one of these sites is the CABP locus, located at 1.p.36, which is linked to increased risk of both prostate cancer and Glioblastoma Multiforme, a brain cancer of presumptive neural stem cell origin (Gibbs et al, 1999) The single human *PERLECAN* Gene also maps to 1.p.36. In addition, several mutations in genes of the SHH pathway have been linked to increased risk of prostate cancer (Datta and Datta, 2006). This raises the possibility that the signaling module already described in *Drosophila* also controls prostate cancer cell proliferation. We hypothesize that PERLECAN modulates SHH signaling and is required for SHH-dependent cell proliferation in advanced prostate cancer.

In a collaborative study, we have shown that *PERLECAN* is necessary for regulation of SHH- dependent proliferation in advanced prostate cancer, and propose a mechanism for how this regulation is achieved using a prostate cancer cell line model (Datta et al., 2006a; Sanchez et al., 2004). In addition, I have evidence supporting a second mechanism for *PERLECAN* regulation of SHH in an alternative cell line model.

Inhibition of prostate cancer proliferation by interference with SONIC HEDGEHOG-GLI1 signaling

Prostate cancer is the most common solid tumor in men, and it shares with all cancers the hallmark of elevated, nonhomeostatic cell proliferation. Here we have tested the hypothesis that the SONIC HEDGEHOG (SHH)-GLI signaling pathway is implicated in prostate cancer. We report expression of SHH-GLI pathway components in adult human prostate cancer, often with enhanced levels in tumors versus normal prostatic epithelia. Blocking the pathway with cyclopamine or anti SHH antibodies inhibits the proliferation of $GLI1^{+}/PSA^{+}$ primary prostate tumor cultures. Inversely, SHH can potentiate tumor cell proliferation, suggesting that autocrine signaling may often sustain tumor growth. In addition, pathway blockade in three metastatic prostate cancer cell lines with cyclopamine or through *GLI1* RNA interference leads to inhibition of cell proliferation,

suggesting cell autonomous pathway activation at different levels and showing an essential role for GLI1 in human cells. Our data demonstrate the dependence of prostate cancer on SHH-GLI function and suggest a novel therapeutic approach.

Introduction

SONIC HEDGEHOG (SHH) signaling has been implicated in different aspects of animal development, acting through several components, including the transmembrane proteins PATCHED1 (PTCH1) and SMOOTHENED (SMOH), to activate the GLI zinc-finger transcription factors (Ingham and McMahon, 2001; Ruiz i Altaba et al., 2002). In addition, we and others have shown that SHH signaling is implicated in a number of tumors (Pasca di Magliano and Hebrok, 2003; Ruiz i Altaba et al., 2002), such as basal cell carcinomas (Dahmane et al., 1997; Hahn et al., 1996; Johnson et al., 1996), medulloblastomas (Berman et al., 2002; Dahmane et al., 2001), gliomas (Dahmane et al., 2001), sarcomas (Hahn et al., 1996; Stein et al., 1999), tumors of the digestive tract (Berman et al., 2003), small cell lung cancers (Watkins et al., 2003), and pancreatic carcinomas (Thayer et al., 2003). To date there is no direct evidence linking SHH signaling to prostate cancer, the most common solid cancer in men (Nelson et al., 2003), although we have found that sporadic prostate tumors express *GLI1* (Dahmane et al., 2001), a reliable marker of SHH signaling (Hynes et al., 1997; Lee et al., 1997). This observation allowed us to propose the hypothesis that the SHH-GLI pathway participates in prostate cancer (Dahmane et al., 2001). Consistently, Shh signaling has been found to be essential for prostate patterning and development (Barnett et al., 2002; Berman et al., 2004; Freestone et al., 2003; Lamm et al., 2002; Podlasek et al., 1999; Wang et al., 2003), and genetic mapping data has revealed that at least two key components of the SHH-GLI pathway [*SMOH* and *SUPPRESSOR OF FUSED* (*SUFUH*)] are located in chromosomal regions implicated in familial human prostate cancer (Easton et al., 2003; Xu et al., 2003). Here we have tested the involvement of SHH-GLI signaling in prostate cancer.

Methods

Cell lines and primary cultures**.** The PC3, LNCaP, and DU145 cell lines (Horoszewicz et al., 1980; Kaighn et al., 1978; Stone et al., 1978) were purchased from American Type Culture Collection and grown as specified. All primary prostate tumors were obtained following approved protocols. Tumors in PBS were chopped with a razor blade and incubated with Papain for 1 h at 37°C, they were then dissociated by passing them through a fire-polished pipette and washed several times in serum containing media. All dissociated primary tumors were plated in polyornithin and laminin-treated p16 plates in DMEM-F12 with 10% FBS at ~30,000 cells per p16 well. Primary cultures were used 2-4 days after plating, when the cells reached 60-70% confluence.

In situ hybridization and immunocytochemistry. Immunocytochemistry was performed with anti-BrdUrd (Beckton Dickinson), anti- SHH (Santa Cruz Biotechnology), and anti-Ki-67 (DAKO), using FITC- or horseradish peroxidase (HRP)-conjugated secondary antibodies (Boehringer Mannheim) as described (Dahmane et al., 2001). For tissue arrays, slides were baked and deparaffinized before blocking of endogenous peroxides. They were then developed with HRPconjugated secondary antibodies and diaminobenzidine (DAB). *In situ* hybridizations on frozen sections with digoxygenin-labeled antisense RNA probes for *GLI1*, *PTCH1*, and *SHH* and a sense control *GLI1* were as described (Dahmane et al., 2001).

Prostate tissue microarrays and microdissection. After institutional review board approval, tissue microarrays (Matysiak et al., 2003) were prepared from archived paraffin blocks from 288 radical prostatectomy cases from the Medical College of Wisconsin. For each case, 0.6-mm cores of tumor were isolated and placed in the array blocks, and 5-μm slides were prepared for immunohistochemistry. Slides were reviewed by a trained urologic pathologist (M.W.D.) and scored for the presence of benign

prostate glands, high-grade prostatic intraepithelial neoplasia, or invasive tumor. The presence of tumor or high-grade prostatic intraepithelial neoplasia was confirmed by immunohistochemical staining for high molecular mass cytokeratin (CK903 Ab, DAKO). Individual cores were examined as duplicates, and staining was correlated to a set of anonymous deidentified pathologic and outcomes data with χ^2 and Fisher's exact or two-tailed ANOVA analyses.

Normal and tumor tissue from the same patients for real-time PCR analyses were microdissected from sections with a laser capture microscope after pathological assessment.

SHH, anti-SHH antibody, cyclopamine, and tomatidine treatments. Commercial N-SHH (R & D Systems) was used at 100 nM because we have found that this commercial protein is ~20 times less active than the octyl-modified SHH-N we had previously used from Curis in the C3H10T1/2 induction assay (data not shown). 5E1 anti-SHH blocking antibody (Ericson et al., 1996) was purchased from the Hybridoma Bank at the University of Iowa and was used at 8 μg/ml. Cyclopamine (Toronto Research Chemicals) and Tomatidine (Sigma) were used at 10 μM unless otherwise noted; for cells in culture, they were dissolved in ethanol, and ethanol alone was used as control. Treated cells were in 2.5% serum for 48 h instead of the usual 10% routinely used for standard growth.

Proliferation assays. BrdU (Sigma) was given at 4 μg/ml before fixation. The time of the BrdU pulse depended on the growth rate of the cells tested. Cell lines were given a 2-h pulse, whereas primary tumor cultures, which grow less rapidly, were given 16-h pulses. Proliferation in tissue arrays was measured by the level of Ki-67 antigen expression.

PCRs. For RT-PCRs, the following primers were used (all 5′ to 3′). GLI1s, GGGATGATCCCACATCCTCAGTC, and GLI1a, CTGGAGCAGCCCCCCCAGT at

60°C; PSAs, CTTGTAGCCTCTCGTGGCAG, and PSAa, GACCTTCATAGCATCCGTGAG at 56°C. Primers for *PTCH1* and *GAPDH* were as described (Dahmane et al., 2001; Palma and Ruiz i Altaba, 2004).

For real-time PCR, total RNA was DNase treated (Invitrogen) and reverse transcribed with TaqMan (Applied Biosystems) using oligo(dT) primers as described by the manufacturer. Reactions were run by using SYBR Green (Applied Biosystems) on an ABI Prism 7700 machine. Each sample was run minimally at three concentrations in triplicate. All primer sets amplified 75- to 300-bp fragments. Sequences are available upon request. The raw data are available upon request from S.Datta.

RNA interference. Double-stranded small interference RNAs (siRNAs, 21 nt long) were purchased from Dahrmacon, purified, and desalted. The sequences for the GLI1 siRNAs used was: AACUCCACAGGCAUACAGGAU; control siRNA was: AACGUACGCGGAAUACAACGA. This siRNA was also used FITC tagged. siRNA transfections (0.2 μM) were with Oligofectamine (Invitrogen) as described by the manufacturer. Cells were treated for 60 h before fixation.

Results

To begin to analyze the role of SHH-GLI signaling in prostate cancer, we first tested for the expression of SHH-GLI pathway components in prostate cancer resections and normal tissue from the same patients. *In situ* hybridization showed that *GLI1*, *PTCH1*, and *SHH* are normally coexpressed in epithelial cells and not in the surrounding stroma (Figure 3.1 *A*, *C*, *E*, *G*, *I*, *L*, and *O*). Prostate tumors were uniformly $SHH^{+}/GLI1^{+}/PTCH1^{+}$ (Figure 3.1 *B*, *D*, *F*, *H*, *J*, *K*, *M*, *N*, *P*, and *Q*), although variable levels of expression were detected visually in the tumors. Coexpression of these markers in tumor cells is consistent with their derivation from the normal prostatic epithelium.

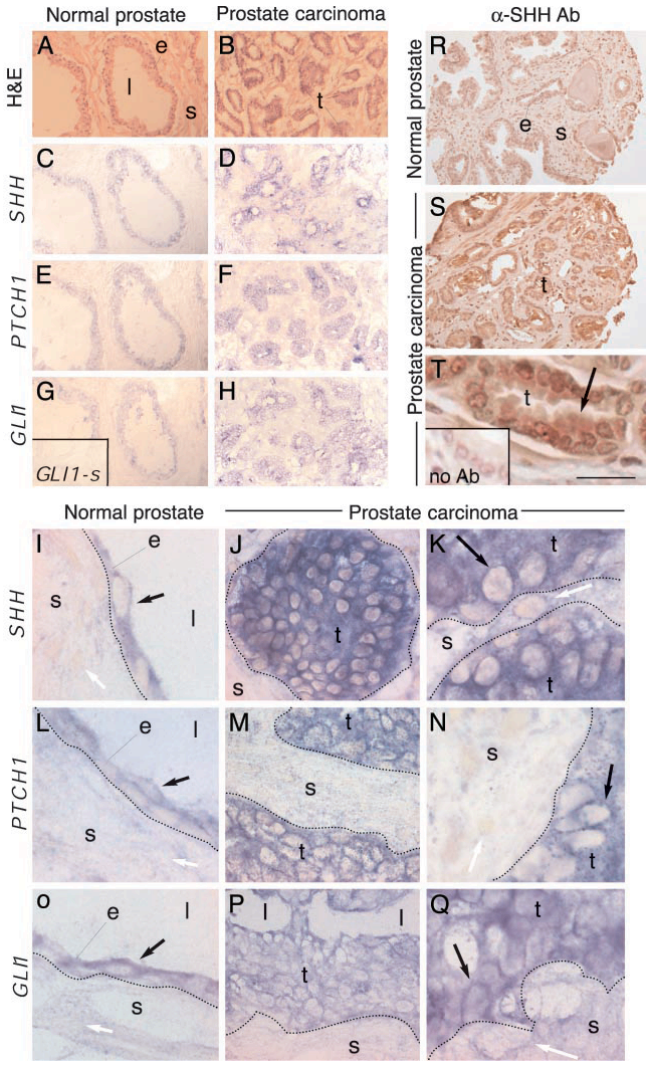


Fig.3.1. Expression of SHH–GLI pathway components in normal prostate tissue and prostate tumors. Sections of normal prostate tissue (*A*, *C*, *E*, *G*, *I*, *L*, and *O*) and prostate tumors (*B*, *D*, *F*, *H*, *J*, *K*, *M*, *N*, *P*, and *Q*) show hematoxylin and eosin (H & E) staining (*A* and *B*) or the expression of *SHH* (*C*, *D*, and *I-K*), *PTCH1* (*E*, *F*, and *L-N*), and *GLI1* (*G*,*H*, and *O-Q*). (*GInset*) Sense *GLI1* probe control showing no background. Prostate tumors havemanysmall epithelial glandular structures. Black arrows point to expressing cells. White arrows point to nonexpressing cells. (*R-T*) Sections from the tissue microarrays of normal prostate tissue (*R*) and prostate tumors (*S* and *T*) showing expression of SHH protein with an anti-SHH antibody (αSHH Ab) (*R-T*) and a no primary antibody control (*T Inset*). All sections were counterstained with hematoxylin to visualize nuclei and tissue structure. Arrow in *T* points to localization of SHH protein in the cytoplasm of epithelial cells. e, epithelium; l, lumen; s, stroma; t, tumor. (Scale bar in *T* is 150 μm in *A-H*, *R*, and *S*, 20 μm in *J*, *M*, *P*, and *T*, and 10 μm in *I-L*, *N*, *O*, and *Q*.)

Table 3.1
SHH, GLI1, GLI2, GLI3, and PTCH1 expression in human prostate cancer
Fold increase in tumors versus matched normal tissue determined by real-time RT-PCR analyses as calculated by the ΔCT method. Range indicates1standard deviation. Gene expression levels were normalized to β-actin. Increases of 2fold or more are shown in bold.

	SHH		*PTCH1*		*GLI1*		*GLI2*		*GLI3*	
Patient	Fold Increase	Range	Fold Increase	Range	Fold Increase	Range	Fold Increase	Range	Fold Increase	Range
829	0	0–0.01	1.5	1.1–2.1	26.1	20.8–32.7	0.02	0.02–0.02	72	53–99
887	0.2	0.05–0.9	8.5	7.6–9.5	0.09	0.07–0.13	0.43	0.37–0.51	1.1	0.8–1.6
921	2.9	1.3–6.3	50	30–84	2	1.1–3.4	3.8	2.4–6.1	12.5	7.7–20.4
945	9.8	6.2–15.7	7.8	5.7–10.7	22.7	21.6–23.9	0.7	0.5–1.0	2.2	2.1–2.4
1854	4.7	1.8–11.7	213	164–278	5.1	3.8–6.9	19.5	10.9–35.1	5.7	4.4–7.5
1866	4.6	4.1–5.2	3.4	3.1–3.7	299	260–342	0.03	0.02–0.03	0.18	0.15–0.2

More sensitive real-time PCR analyses of six of the same microdissected matched pairs showed up-regulation of the expression of *SHH*, *PTCH1*, *GLI1*, *GLI2*, and *GLI3* (between 1.5- and ~300- fold) in many tumor cases compared to normal tissue after normalization to the ubiquitous similar expression of *β-actin* (Table 3.1). Levels of expression within tumors were variable. Such differences could be related to the known heterogeneity of prostate cancer, because this is a general diagnosis that encompasses a broad range of histological phenotypes (Bostwick et al., 2004; DeMarzo et al., 2003; Kaplan-Lefko et al., 2003). Whereas varying levels have also been observed in other tumors (Ingham and McMahon, 2001; Ruiz i Altaba et al., 2002), the meaning of such differences is not known, although they have been proposed to correlate in a direct or inverse manner with tumor type or grade (Grachtchouk et al., 2003; Katayam et al., 2002; Pomeroy et al., 2002). What is important is that the loyal markers of an active SHH-GLI pathway, *GLI1* and *PTCH1* (Goodrich et al., 1996; Lee et al., 1997; Podlasek et al., 1999), are consistently transcribed in the examined tumor cells, showing the presence of an active pathway.

To extend these findings, we performed immunohistochemistry for SHH, as a secreted and potentially useful systemic marker for prostate cancer, on tissue microarrays representing 239 prostate carcinomas, 15 precancerous lesion high-grade prostatic intraepithelial neoplasia (HGPIN), and 135 benign prostate tissues from 297 patients. SHH expression was increased in tumors and was present as a secreted protein in the glandular lumens made by tumor cells (Figure 3.1 *R-T*), likely reflecting the origin of tumors from the SHH^+ prostatic epithelia. Higher SHH levels, determined visually, were found in 33% of tumors compared to <1% of cases of normal adjacent tissue, indicating a significant correlation between high SHH levels and tumor presence. High SHH levelswere also correlated with higher Ki-67^+ cell proliferation (Table 3.2). The level of SHH expression was not correlated with Gleason score or other clinical parameters (Table 3.2). This finding may indicate that inappropriately maintained or elevated SHH expression is an early and general event in prostate cancer, reflecting the origin of

Table 3.2
Correlation of elevated SHH expression with tumorigenesis and clinical features of prostate cancer.

		Expression low	Expression high	χ^2 or Fisher's exact test
Histology	Tumor	141	70	
	Normal	126	1	$P < 0.00005$
	HGPIN	13	1	
	Normal	126	1	$P = 0.0563$
Clinical stage	cT2	16	6	
	cT3/4	2	1	NS
Tumor grade	Gleason 6	30	1	
	Gleason 7,8,9	57	7	NS
Pathologic stage	pT1–pT2	50	4	
	pT3	37	4	NS
Nodal status	pN0	27	12	
	pN1	1	0	NS
Outcomes	PSA Recurrence	8	1	
	No PSA recurrence	22	12	NS
Vital status	Alive	42	18	
	Dead	4	3	NS
Ki-67 expression	Sample no.	275	69	
	Mean % Ki-67$^+$ nuclei	5.1	7.6	$P = 0.0141$*

Significance was only found between SHH expression and tumorigenesis and SHH expression and higher proliferative levels as measured by Ki-67 staining. Tumor grade is presented as Gleason score. Pathologic staging uses the American Joint Comission on Cancer 2002 tumor staging criteria. HGPIN, high-grade prostatic intraepithelial neoplasia.
*Two-tailed ANOVA.

tumors from the SHH^{+} prostatic epithelia.

The difficulty of growing human prostate cancer cells *in vitro* translates into a dearth of available cancer cells to test. Here we have chosen the three most widely used prostate cancer cell lines, LNCaP, an androgen sensitive cell line derived from a prostate cancer lymph node metastasis; and PC3 and DU145, androgen insensitive cell lines derived from prostate cancer bone metastases, to assay for the expression of SHH-GLI pathway components. All of the cells expressed *GLI1* and *PTCH1* (Figure 3.2*A*), consistent with our expression studies and indicating that they harbor an active pathway. Of these cell lines, only DU145 and PC3 cells expressed *GLI2*, and only LNCaP and PC3 cells expressed *GLI3* and *SHH* at detectable levels (Figure 3.2*A*). *GLI1* is thus the only *GLI* gene consistently expressed at detectable levels in all of these cells, and thus, we have focused on *GLI1*.

To interfere with SHH-GLI signaling, we first used cyclopamine, a selective inhibitor of SMOH (Chen et al., 2002). Effects of cyclopamine treatment after 48 h were tested by BrdUrd incorporation as a sensitive measure of cell proliferation. Such treatment led to a large (>80%) decrease in BrdUrd incorporation in LNCaP cells, and a significant decrease (≈30%) in PC3 cells but had no effect in DU145 cells (Figure 3.2*B*). Treatment with tomatidine (Chen et al., 2002)served as control and had little or no effect on BrdUrd incorporation (Figure 3.2*B*). The lack of effects of cyclopamine on DU145 cells shows that this drug is not nonspecific. Because we used short-term assays to focus on early, direct effects on cell proliferation, the changes in total cell number were consequently relatively conservative. For instance, cyclopamine reduced total 4′, 6-diamidino-2-phenylindole-positive LNCaP cell number by 22.1±1.1% (P=0.0001) after 48 h. No cytotoxic effects or significant cell death were observed during these experiments. Cyclopamine treatment also led to a decrease in *GLI1* expression, consistent with the expected down-regulation of the SHH-GLI pathway (Figure 3.2*C*).

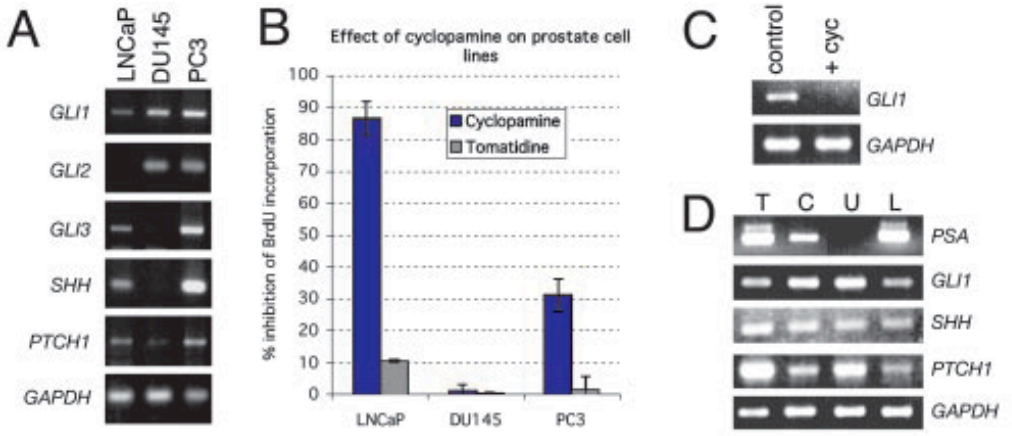


Fig. 3.2. Response of prostate tumor cell lines to alterations in the SHH-GLI1 pathway. (*A*) PCR analyses for the expression of SHH-GLI pathway components in three cell lines as indicated. In this and all other PCR assays, the expression of the ubiquitous gene *GAPDH* is measured as quantitative control. (*B*) Inhibition of prostate cell line proliferation as measured by BrdUrd incorporation in the three prostate cell lines used with cyclopamine. Tomatidine is used as control. (*C* and *D*) PCR analyses of the suppression of *GLI1* expression in LNCaP cells by cyclopamine treatment at 36 h (*C*) or of the expression of prostate specific antigen (PSA), *GLI1*, *SHH*, and *PTCH1* expression in whole prostate tumor tissue (T), primary culture (C), the glioblastoma cell line U87 (U), and LNCaP (L) cells (*D*). *PSA* is expressed in prostate but not in brain cells. All samples express *GLI1* and *SHH*. The whole tissue and primary culture correspond to PT6.

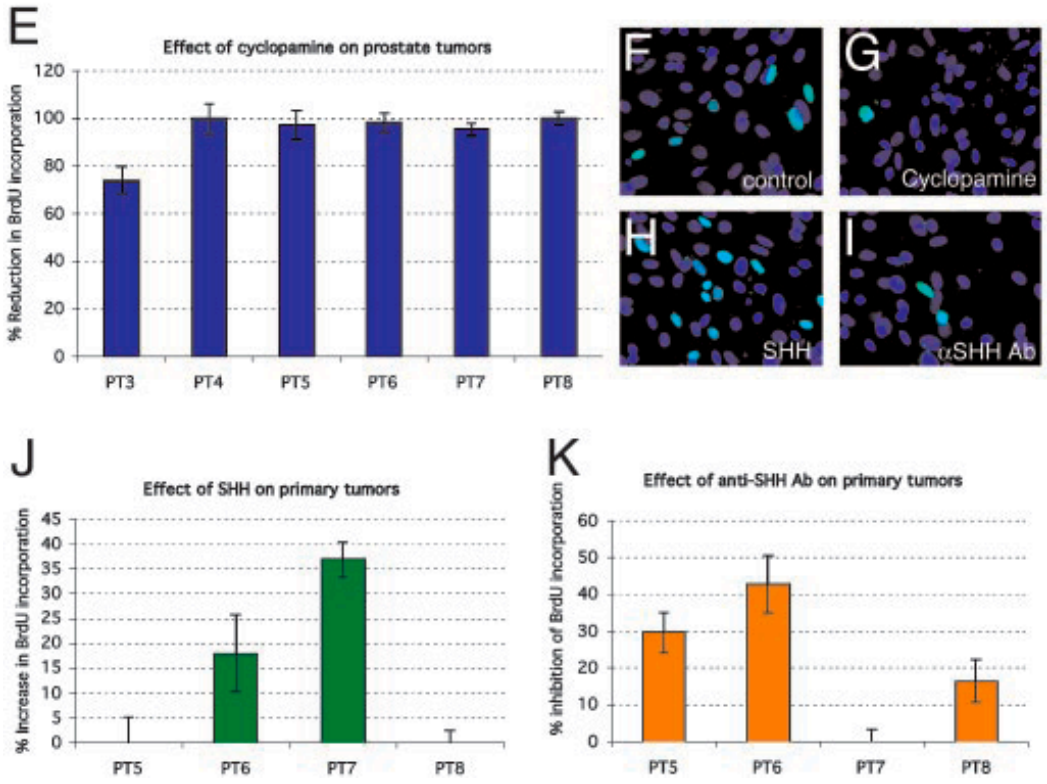


Fig. 3.2. Continued. (*E*) Histogram of the inhibition of BrdUrd incorporation in primary cultures of prostate tumor (PT3-PT8) by cyclopamine treatment. (*F-I*) Immunocytochemistry for BrdUrd incorporation with secondary FITC antibodies showing $BrdUrd^{+}$ nuclei (green) in a field of primary prostate cells (PT6) in control cells (treated with ethanol as the carrier for cyclopamine, *F*), cyclopamine (*G*), SHH protein (*H*), or anti-SHH antibody (αSHH Ab, *I*). All nuclei are stained with 4′, 6-diamidino-2-phenylindole (blue). (*J* and *K*) Histograms of the increase in (*J*) or inhibition of (*K*) BrdUrd incorporation of primary prostate tumors after treatment with SHH (*J*) or anti-SHH antibody (αSHH Ab, *K*) for 48 h. Histogram error bars represent SEM in all panels.

Analyses of primary prostate tumors is complicated by the difficulty of growing primary human prostate cancer cultures (Rhim, 2000). Nevertheless, we were able to dissociate and plate six of eight primary prostate tumors, although stable cultures were not obtained. Primary cells that remained attached after 2 days had a uniformcuboidal morphology, formed small clusters and expressed prostate-specific antigen (PSA), as well as *SHH*, *PTCH1*, and *GLI1* (Figure 3.2*D*), proving their prostatic epithelial origin. Cyclopamine treatment led to a major (>70%) decrease in BrdUrd incorporation in all primary cultures as compared with carrier-treated samples (Figure 3.2*E*-*G*), mimicking the results obtained in LNCaP cells. Here again, the insensitivity of DU145 to cyclopamine provides a control for the action of the drug. Indeed, although we have not tested the response of normal human prostate cells to cyclopamine, we expect that it would also inhibit the proliferation of normal SHH^+/ $PTCH1^+$/$GLI1^+$ prostate epithelial cells (Figure 3.1). As with the cell lines, the total number of 4′, 6-diamidino-2-phenylindole-positive primary tumor cells was similarly reduced by cyclopamine treatment [e.g., 26.7 ± 1.1% decrease in primary tumor 6 (PT6), $P = 0.001$] after 48 h. Although stromal cells are likely to be present in our primary cultures, their numbers appear to be small because >90% of the cells examined microscopically had a similar cuboidal morphology. Moreover, the high inhibition levels by cyclopamine would be inconsistent with effects only in contaminating stromal cells, which do not appreciably express *PTCH1* or *GLI1* (Figure 3.1).

We then tested for the ability of exogenous SHH to stimulate prostate cancer cell proliferation and for the possible existence of autocrine signaling. Addition of recombinant SHH protein led to an increase in BrdUrd incorporation in two of four primary cultures after 48 h (Figure 3.2 *F*, *H*, and *J*). In contrast, addition of the standard blocking antibody against SHH (5E1; (Ericson et al., 1996)) resulted in an inhibition of BrdUrd incorporation by 15-40% for three of four tumors (Figure 3.2 *F*, *I*, and *K*), suggesting that several tumors display autocrine signaling. Interestingly, the only

primary culture that was insensitive to Shh Ab blockade, PT7, being sensitive to cyclopamine [which targets SMOH (Chen et al., 2002), Figure 3.2 *E*], was also the more sensitive to the addition of exogenous Shh. This might indicate that although the pathway is activated downstream of the site of ligand action in PT7, possibly affecting PTCH1 or SMOH, exogenous Shh can still increase the levels of signaling. Taken together, the functional heterogeneity that we detect parallels that found for *GLI* and *SHH* expression described above and may reflect independent activating events as well as the well known heterogeneity of prostate cancers.

Treatment of LNCaP, PC3 or DU145 cells with either blocking antibody or recombinant Shh protein did not result in significant changes in BrdUrd incorporation (data not shown). LNCaP and PC3 cells could thus display an activated pathway at the membrane level (being sensitive to cyclopamine inhibition) that has lost responsiveness to ligand. Cyclopamine-insensitive DU145 cells may have an activated pathway downstream of SMOH (or at the level of SMOH affecting its inhibition by cyclopamine), having lost also the ability to respond to SHH. It remains possible that the different behavior of primary cultures versus established cell lines also reflects unrelated transformation or immortalization events.

The GLI zinc-finger transcription factors have been suggested to be essential for the mediation of HH signals (Ingham and McMahon, 2001; Ruiz i Altaba et al., 2002; Ruiz i Altaba et al., 2004). However, Gli1 is apparently redundant in mouse development and tumorigenesis (Park et al., 2000; Weiner et al., 2002), and there is to date no data on the requirement for *GLI1* in human cells. Here, we tested the function of GLI1, the only GLI gene consistently expressed in all primary tumors and cell lines, by RNA interference to knockdown its function with a specific 21-nt-long small RNA. (This siRNA inhibits the effect of SHH on multipotent C3H10T1/2 cells; P.S. and A.R.A., unpublished data). Lipofection of primary cultures resulted in a negligible number of transfected cells, making it impractical to use siRNAs in such cultures. In contrast, lipofection of FITC-

siRNA proved efficient (≈50-80%) in the LNCaP, PC3, and DU145 cell lines (Figure 3.3*A*-*C*). It is important to note that, because transfection efficiencies are <100%, the results of cell pool assays necessarily underestimate the effects of RNA interference. Transfection of a control siRNA at the same concentration served as control in all tests. The specificity of the *GLI1* siRNA was further tested in LNCaP cells. Reduction of *GLI1* mRNA levels by the *GLI1* siRNA was detected as early as 3 h after transfection and at 8 and 24 h, but not at 48 h (Figure 3.3 *D* and *F* and data not shown), suggesting upregulation of *GLI1* after its inhibition, possibly because of the action of a rapid positive feedback loop (Dahmane et al., 2001; Regl et al., 2002). *GLI1* siRNA also robustly repressed *PTCH1*, a result most clearly seen at 48 h, but not the housekeeping gene *GAPDH* (Figure 3.3*D* and data not shown). Because *PTCH1* is a SHH target (Goodrich et al., 1996), and in particular of GLI1 (Agren et al., 2004), this result indicates that interference with GLI1 function by RNAi is selective and effective in prostate cancer cells. *GLI1* siRNA also decreased *GLI1* mRNA levels in DU145 and PC3 cells after 8 h (Figure 3.3*F*).

Inhibition of GLI1 by RNA interference led to a variable reduction in BrdUrd incorporation in all three cell lines, with strongest effects (≈60%) in LNCaP cells (Figure 3.3*E*). These cells are thus very sensitive to inhibition by cyclopamine and *GLI1*interference, suggesting the presence of a fully active canonical pathway activated at the level of SMOH or upstream, but downstream of SHH, because treatment with the blocking anti-SHH Ab had no effect. DU145 cells are not sensitive to cyclopamine, but are sensitive to *GLI1* interference, suggesting activation downstream of SMOH and upstream or at the level of GLI1 function. In contrast, PC3 cells are sensitive to cyclopamine and less so to *GLI1* interference, perhaps suggesting compensation by the other GLI proteins because PC3 cells express *GLI2* [and this *GLI* gene mediates SHH signals (Roessler et al., 2003) and can behave like Gli1 in mice (Bai and Joyner, 2001)] or the presence of alternate pathways for tumor cell proliferation. We note, however, that lipofection efficiencies in PC3 cells (Figure 3.3*C*) are the lowest (≈50%) of the three

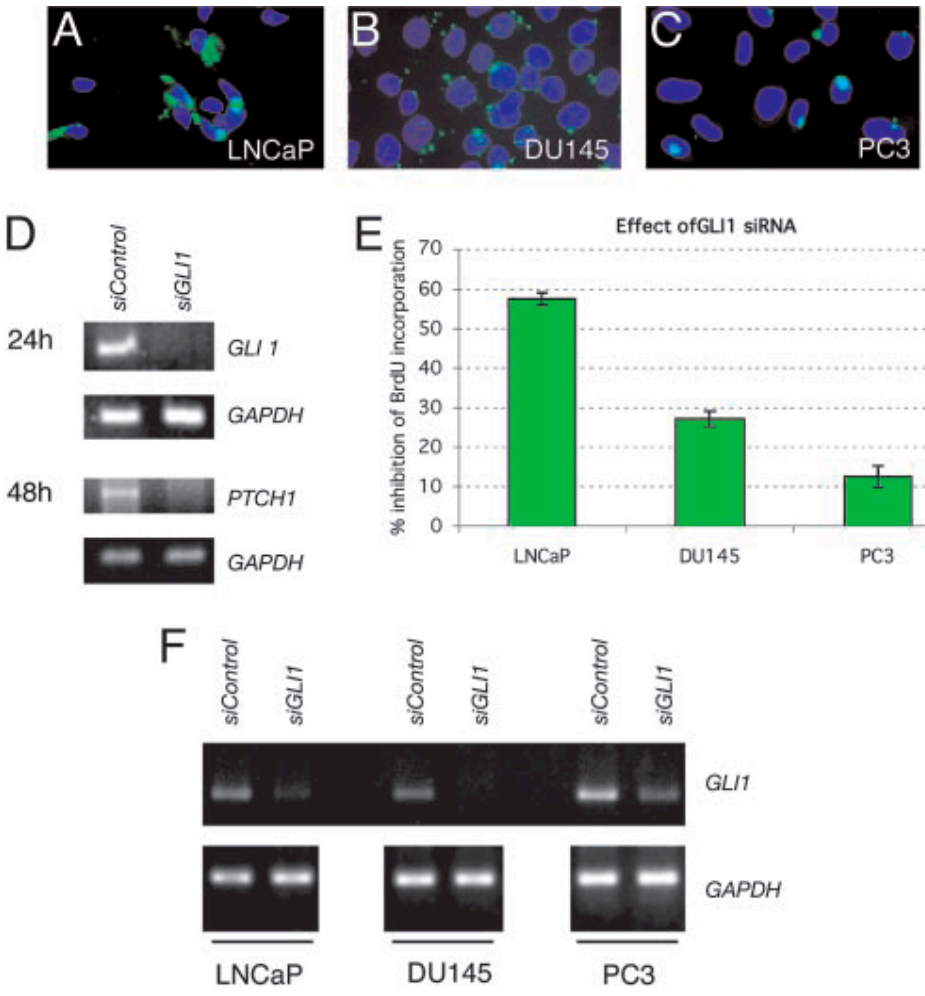


Fig.3.3. Response of prostate cell lines to *GLI1* RNA interference. (*A-C*) Immunocytochemisty of the three prostate cell lines indicated showing the efficiency of lipofection of an FITC-tagged control siRNA (green). Note the lower efficiency in PC3 cells. (*D*) Effect of *GLI1* siRNA on gene expression. RNA interference reduces *GLI1* and *PTCH1* mRNA levels as seen at 24 and 48 h, respectively (*E*) Histogram of the inhibition of BrdU incorporation in prostate tumor cell lines by *GLI1* siRNA. (*F*) Specificity of the effects of *GLI1* siRNA on *GLI1* mRNA levels in the three prostate cell lines, compared with those of a control unrelated siRNA, 8 h after transfection. The levels of *GAPDH* are shown below as controls.

cells tested, indicating that the real effects of *GLI1* interference may be higher. Taken together, our results show the requirement of GLI1 in human prostate tumor cells.

Discussion

Here we demonstrate the dependence of prostate cancer cell proliferation on SHH-GLI pathway activity. The data suggest activation of the pathway at different levels in primary prostate tumors and cell lines derived from metastatic lesions. These findings, together with the involvement of this pathway in normal prostate development and growth (Barnett et al., 2002; Berman et al., 2004; Freestone et al., 2003; Lamm et al., 2002; Podlasek et al., 1999; Wang et al., 2003), indicate that the normal patterning role of SHH-GLI signaling is deregulated in cancer. This idea is consistent with the proposed events in other tissues, including brain, lung, stomach, muscle, pancreas, and skin, in which the SHH-GLI pathway regulates patterned growth and when deregulated can give rise to SHH-GLI dependent tumors (Barnett et al., 2002; Berman et al., 2004; Freestone et al., 2003; Lamm et al., 2002; Pasca di Magliano and Hebrok, 2003; Podlasek et al., 1999; Ruiz i Altaba et al., 2002; Wang et al., 2003). Thus, there is a surprising and unexpected parallel in the requirement of SHH-GLI signaling of prostate tumors with those in organs of very different origin, function, and location.

The deduction that prostate tumors display activation at different levels is consistent with findings in brain ((Dahmane et al., 2001)and P.S. and A.R.A., unpublished data) and pancreatic (Nelson et al., 2003) tumors, even though the entire set of activating events or mutations have not been described in any case. Indeed, our data suggest that the regulation of the SHH-GLI pathway in the normal prostatic epithelium is altered away from homeostasis in the tumors by epigenetic events or mutations in components such as PTCH1, SMOH, or SUFUH, similar to those already found in other tumors (e.g., (Dong et al., 2000; Pietsch et al., 1997; Raffel et al., 1997; Reifenberger et al., 1998; Taylor et al., 2002; Wolter et al., 1997; Zurawel et al., 2000)). However, the finding that

the pathway is active as assessed by the expression of *GLI1* and *PTCH1* [as in the case of basal cell carcinomas (Dahmane et al., 1997), medulloblastomas (Dahmane et al., 2001) and gliomas (Dahmane et al., 2001)] allows us to bypass the identification of the likely myriad of activating events to discern that tumor cells harbor an active pathway. Indeed, the finding that SHH expression levels are not correlated with Gleason score, but that all prostate tumor samples tested require continued pathway activity for proliferation, allows us to propose that this pathway is a critical and essential component of prostate cancer.

Specifically, we show the requirement for SHH, SMOH, and/or GLI1 for the proliferation of prostate cancer cells. The fact that all primary tumors tested are sensitive to cyclopamine indicates that SMOH, or upstream elements from it, are common targets leading to the activation of downstream mediators. Several primary cultures are also sensitive to inhibition by blocking anti-SHH Ab, suggesting that, like in stomach tumors (Berman et al., 2003), autocrine signaling is a frequent cause of pathway activation in prostate cancer. The consistent expression of *GLI1* in tumor cell lines and in primary tumors together with the effects of RNA interference indicate that this *GLI* gene plays a central and general role in prostate tumor cell proliferation, and demonstrate its requirement in human tumorigenesis. In contrast, *GLI2* and *GLI3* do not appear to be consistently expressed in prostate cancer cells. When expressed, they could have complementary or compensatory roles in some cases, although their roles remain to be determined.

Prostate cancer is thought to develop from a lesion in the epithelial layer to become an invasive tumor that spreads within the prostate and subsequently acquires the potential to metastasize to distant sites, most often the lymph nodes and bone (Abate-Shen and Shen, 2000). Inhibition of testosterone-dependent tumor growth is the common treatment for advanced disease, but subsequent hormone-independent cell proliferation and metastasis often leads to patient death (Martel et al., 2003). Our data on the behavior of the three

prostate cancer cell lines derived from metastatic lesions suggest that such tumors could harbor additional changes that may make them ligand-independent, albeit still being SHH-GLI pathway dependent, and explain their differential behavior in comparison with the primary cultures. Perhaps the gain of intracellular, cell-autonomous activation of the SHH-GLI pathway represents an advantage for metastatic cells, allowing efficient proliferation far from the prostatic epithelium, where SHH appears to be continually and abundantly produced.

The high inhibition of proliferation by SHH-GLI pathway blockade of the presumed androgen-sensitive primary tumors used in this study, which derive from patients that did not receive hormone treatments, and of the androgen sensitive LNCaP cell line might be related to the proposed requirement of Shh signaling for normal androgen function, because defects derived from loss of Shh signaling in mice can be rescued by exogenous androgens (Berman et al., 2004). Prostate cancer could therefore initiate through inappropriate maintenance or enhanced activity of SHH-GLI signaling, and more aggressive (androgen insensitive) states may require additional alterations. Nevertheless, the inhibition of the androgen-insensitive DU145 cell line by RNA interference suggests that even highly aggressive tumors may be sensitive, albeit to different degrees, to GLI1 inhibition.

Prostate stem cells may play a critical role in the epithelial development and homeostasis (Bonkhoff, 1996; De Marzo et al., 1998). Because cancer may be a disease of stem cell lineages (discussed in (Pasca di Magliano and Hebrok, 2003; Reya et al., 2001; Ruiz i Altaba et al., 2002; Ruiz i Altaba et al., 2004)) and SHH-GLI signaling controls the behavior of precursors and of cells with stem cell properties in the mammalian brain (e.g., (Lai et al., 2003b; Machold et al., 2003; Palma and Ruiz i Altaba, 2004)and V. Palma, D. Lim, N. Dahmane, N., P.S., Y. Gitton, A. Alvarez-Buylla, A., and A.R.A., unpublished data) and in other tissues and species (Park et al., 2003; Zhang and Kalderon, 2001)), prostate cancer might derive from inappropriate expansion of prostatic

epithelial stem cell lineages caused by abnormal SHH-GLI function.

Finally, our data suggest that SHH and GLI1 may not only be useful markers for prostate cancer but also good targets for anticancer therapies, with emphasis on GLI function as the last and essential step of the pathway, the inhibition of which will likely block signaling by upstream events at any level. SHH-GLI pathway blocking agents should thus provide attractive therapeutic strategies to combat prostate cancer of any grade.

***PERLECAN,* a candidate gene for the CAPB locus, regulates prostate cancer cell growth via the SONIC HEDGEHOG pathway** [*]

Genetic studies associated the CAPB locus with familial risk of brain and prostate cancers. We have identified *HSPG2* (*PERLECAN*) as a candidate gene for CAPB. Previously we have linked PERLECAN to Hedgehog signaling in *Drosophila*. More recently, we have demonstrated the importance of Hedgehog signaling in humans for advanced prostate cancer. Here we demonstrate *PERLECAN* expression in prostate cancer, and its function in prostate cancer cell growth through interaction and modulation of Sonic Hedgehog (SHH) signaling. *PERLECAN* expression in prostate cancer tissues correlates with a high Gleason score and rapid cell proliferation. *PERLECAN* is highly expressed in prostate cancer cell lines, including androgen insensitive cell lines and cell lines selected for metastatic properties. Inhibition of *PERLECAN* expression in these cell lines decreases cell growth. Simultaneous blockade of PERLECAN expression and androgen signaling in the androgen-sensitive cell line LNCaP was additive, indicating the independence of these two pathways. *PERLECAN* expression correlates with SHH in tumor tissue microarrays and increased tumor cell

[*] Reprinted with permission from Datta, M.W., Hernandez, A.M., Schlicht, M.J., Kahler, A.J., DeGueme, A.M., Dhir, R., Shah, R.B., Farach-Carson, C., Barrett, A., and Datta, S. 2006. *PERLECAN*, a candidate gene for the CAPB locus, regulates prostate cancer cell growth via the Sonic Hedgehog pathway. Mol Cancer 5, 9.

proliferation based on Ki-67 immunohistochemistry. Inhibition of *PERLECAN* expression by siRNA in prostate cancer cell lines decreases SHH signaling while expression of the downstream SHH effector *GLI1* rescues the proliferation defect. PERLECAN forms complexes with increasing amounts of SHH that correlate with increasing metastatic potential of the prostate cancer cell line. SHH signaling also increases in the more metastatic cell lines. Metastatic prostate cancer cell lines grown under serum-starved conditions (low androgen and growth factors) resulted in maintenance of *PERLECAN* expression. Under low androgen, low growth factor conditions, *PERLECAN* expression level correlates with the ability of the cells to maintain SHH signaling. We have demonstrated that PERLECAN, a candidate gene for the CAPB locus, is a new component of the SHH pathway in prostate tumors and works independently of androgen signaling. In metastatic tumor cells increased SHH signaling correlates with the maintenance of PERLECAN expression and more PERLECAN-SHH complexes. PERLECAN is a proteoglycan that regulates extracellular and stromal accessibility to growth factors such as SHH, thus allowing for the maintenance of SHH signaling under growth factor limiting conditions. This proteoglycan represents an important central regulator of SHH activity and presents an ideal drug target for blocking SHH effects.

Background

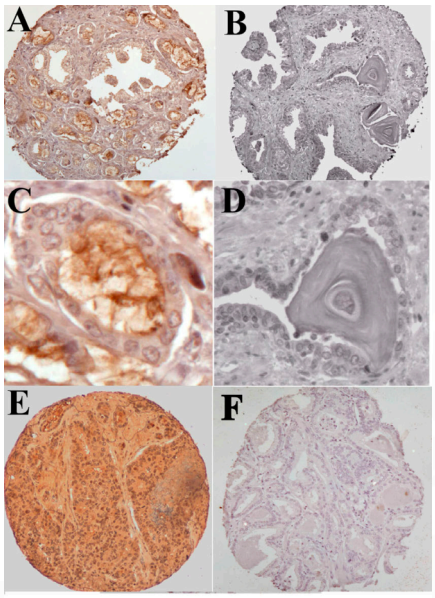
Genetic mapping studies for familial prostate cancer have identified numerous chromosomal regions linked to prostate cancer susceptibility. On chromosome one a genetic association has been demonstrated between clinically significant prostate cancer and the brain tumor glioblastoma multiforme at 1p36 (CArcinoma Prostate Brain, CAPB), suggesting the presence of a common oncogene for these tumors (Conlon et al., 2003; Gibbs et al., 1999; Janer et al., 2003; Park et al., 2003; Zhang and Kalderon, 2001). Using bioinformatics based analysis of text mining and gene expression data we have identified candidate genes within the CAPB locus. One of these genes is HSPG2

(PERLECAN). PERLECAN is a heparan sulfate proteoglycan that is secreted into the extracellular matrix and can bind growth factors (Iozzo et al., 1994). Thus PERLECAN can act as a reservoir or modulator of growth factor function. One growth factor associated with PERLECAN is Hedgehog (Park et al., 2003). SHH signaling has recently been shown to be critical for cancer growth and metastasis in multiple tumor types (Datta and Datta, 2006)In a large proportion of prostate cancers high levels of *SHH* expression is observed along with expression of multiple members of the Hedgehog signaling pathway such as its receptor *Patched1*, downstream transcription factor *Gli1*, and intracellular modulator *Hedgehog Interacting Protein* (Sanchez et al., 2004; Sheng et al., 2004). Activation of the Hedgehog pathway has been detected in metastatic prostate tumors (Karhadkar et al., 2004; Sheng et al., 2004), and higher levels of pathway activity are associated with the metastatic phenotype (Karhadkar et al., 2004). Blocking the SHH pathway with cyclopamine inhibits proliferation of prostate cancer cell lines (Karhadkar et al., 2004; Sanchez et al., 2004; Sheng et al., 2004) and primary prostate tumor cell cultures (Sanchez et al., 2004). Treatment of mice with cyclopamine results in the inhibition of tumor xenograft growth in multiple tumor types, including prostate tumors (Berman et al., 2003; Sanchez et al., 2004). Our bioinformatics analyses (Datta and Datta, 2006; Sanchez et al., 2004) suggested that genes encoding two components of the SHH pathway, *Suppressor of Fused* (*Su(fu)*) and *Smoothened*, the target of cyclopamine, lie in chromosomal regions implicated in familial prostate cancer (Easton et al., 2003; Xu et al., 2003). Su(fu) is a negative regulator of pathway activity, thus loss of Su(fu) function would increase SHH activity. Molecular analyses of prostate tumors revealed that Su(fu) protein is absent in most highly aggressive tumors and somatic truncation mutations in the *Su(fu)* gene have been identified (Sheng et al., 2004) consistent with the hypothesis that *Su(fu)* would act as a prostate tumor suppressor gene by inhibiting SHH signaling. These studies demonstrate the critical nature of SHH signaling in tumorigenesis and metastasis. Thus identification of additional mechanisms for the regulation of SHH signaling in cancer takes on added importance. Here we demonstrate that expression of the candidate CAPB gene *HSPG2* (*PERLECAN*) is

present in prostate cancers, up-regulated in aggressive prostate cancers and under poor cell growth conditions, and regulates prostate cancer cell proliferation. In addition, we demonstrate that PERLECAN's effects on cell growth are independent of androgen signaling and occur through the binding of SHH, resulting in modulation of the SHH-Patched-Gli signaling pathway. This data, along with data linking PERLECAN to metastatic tumor environments such a bone matrix (Savore et al., 2005), presents a general model in which *PERLECAN* expression by tumor cells under poor growth conditions enhances their ability to utilize growth factors until their spread to suitable metastatic tumor microenvironments for accelerated growth.

Results

PERLECAN is expressed in and associated with aggressive prostate cancers. After identification of *PERLECAN* as a candidate gene for the CAPB locus we sought to confirm the presence of PERLECAN in primary prostate cancers. Immunohistochemical analysis for PERLECAN in prostate cancer tissue microarrays with 600 patient samples demonstrated that PERLECAN, a secreted proteoglycan, is present in the lumens of 54% of malignant prostate cancer glands, but not in normal glands (Figure 3.4A–D, Table 3.3). There was a significant increase in PERLECAN levels in invasive tumors compared to either benign prostate tissue or the precancerous lesion high grade prostatic intraepithelial neoplasia (HGPIN). In particular *PERLECAN* expression was associated with more aggressive tumors, as evidenced by their higher Gleason score (Gleason score 7,8,9 versus Gleason score 5 and 6 tumors). *PERLECAN* expression was also significantly associated with increased prostate cancer cell proliferation, as demonstrated by Ki-67 (PCNA) Immunohistochemical staining (Table 3.3). To extend the evaluation of PERLECAN we examined *PERLECAN* RNA (Figure 3.4G) and/or protein (Figure 3.4H) levels in matched benign and tumor samples from 10 individual patients. At the RNA level *PERLECAN* was significantly increased in four out of six matched patient tumor and benign prostate samples. PERLECAN protein was upregulated in two of four



G

Patient	Fold change in Perlecan mRNA expression	Range of Range of mRNA change	Gleason Grade
945	4.2	4.0-4.4	6
1854	595	550-640	6
921	2.6	1.5-4.3	6
829	0.08	0.06-0.11	7
887	0.20	0.1-0.3	7
1863	2.3	1.5-3.5	8

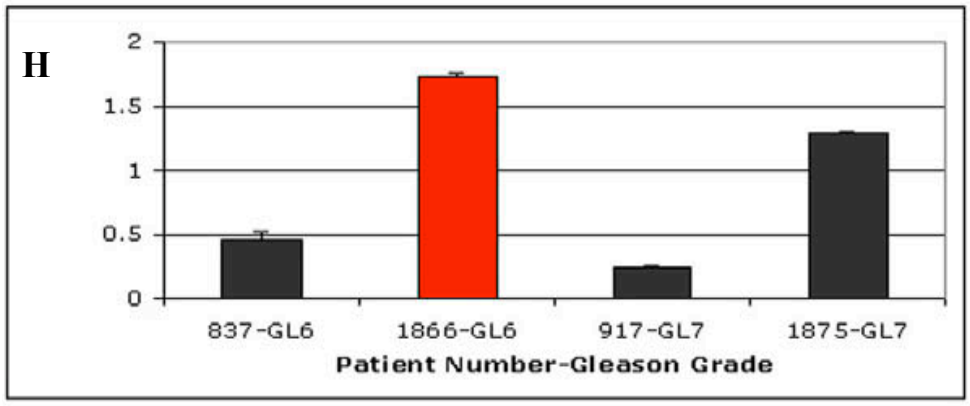


Fig.3.4. PERLECAN protein levels in human prostate tumors. Immunohistochemistry of PERLECAN protein in prostate cancer (A) and normal prostate (B). PERLECAN is present as a secreted protein in the tumor gland lumens (C) but not in the lumens of benign glands or benign corpora amylacea secretions (D). Staining is also seen in metastatic prostate cancer specimens (E). Secondary antibody alone control fails to demonstrate staining (F). All images originally photographed at 400 X magnification. Quantitation of PERLECAN mRNA expression by Real Time PCR (G) or protein by digitized dot blot (H) in normal prostate and tumor samples from individual patients presented as fold change in tumor versus normal. Gleason scores for the tumors are listed. Red numbers or columns indicate patients previously shown to have increased expression of *SHH*, *PTCH1* and *GLI1* (Dahmane et al., 2001).

Table 3.3.
Immunohistochemical staining for PERLECAN and colocalization with Ki-67.

		Perlecan Negative	Perlecan Positive	
Histology	Tumor	170	203	
	Normal	211	31	$p < 0.00005$
	Tumor	170	203	
	HGPIN	46	7	$p < 0.00005$
Clinical Stage	cT2	11	12	
	cT3/4	2	3	N.S.
Tumor Grade	Gleason 6	26	5	
	Gleason 7,8,9	38	23	$p = 0.0335$
Pathologic Stage	pT1-pT2	35	17	
	pT3	29	11	N.S.
Nodal Status	pN0	18	23	
	pN1	0	1	N.S.
Outcomes	PSA Recurrence	4	5	
	No PSA Recurrence	13	22	N.S.
Vital Status	Alive	26	36	
	Dead	2	5	N.S.

Perlecan Expression in Metastasis

Metastatic Site	Perlecan Negative	Perlecan Positive	P value vs. Prostate
Primary Tumor (Prostate)	3	24	
Lymph Node	8	9	$p = 0.0073$
Soft Tissue	15	18	$p = 0.0039$
Liver	5	23	$p = 0.4781$
Lung	3	24	$p = 1.000$

Association of Ki-67 (PCNA) Staining with Perlecan Staining

Sample Staining	Number of Samples	Mean % of Ki-67 positivity	Two-tailed ANOVA
Perlecan positive	143	6.715278	$p = 0.0478$
Perlecan negative	214	5.028571	

* Tumor grade is presented as Gleason score. Pathologic staging uses the American Joint Commission on Cancer (AJCC) 2002 tumor staging criteria. HGPIN = high grade prostatic intraepithelial neoplasia.

additional patient samples where protein was examined. An examination of the Gleason score for the primary tumor samples revealed that the only Gleason score 8 tumor upregulated PERLECAN. These findings correlate with the results from the tissue microarrays (Table 3.3). PERLECAN Ki-67 staining was also evaluated in five of the patient samples, two with low PERLECAN, and three with increased PERLECAN expression. Immunoblotting demonstrated a direct correlation between increased PERLECAN expression and increased Ki- 67 levels. These findings matched the Immunohistochemical staining results from the tissue microarrays (Table 3.4). We also examined PERLECAN protein expression on tissue microarray samples from patients with primary and metastatic prostate cancer identified at autopsy. In these samples PERLECAN expression was upregulated in the primary prostate tumor and metastatic prostate cancer that had spread to the lungs and liver (Figure 3.4E, Table 3.4). PERLECAN expression was lower in tumor present in lymph nodes or soft tissue metastasis, indicating site-specific differences in PERLECAN expression in metastatic prostate cancer.

Basal PERLECAN expression is highest in an androgen sensitive tumor cell line. Baseline expression of *PERLECAN* was examined in the metastatic prostate cancer cell lines LNCaP, DU-145, and PC3. Using analysis of spotted cDNA microarray expression data (Schlicht et al., 2004) quantitative Real Time PCR and immunoblotting, *PERLECAN* expression was found in all three cell lines with the highest levels present in the androgen sensitive LNCaP cell line (Figure 3.5A). We extended these findings by examining *PERLECAN* expression with respect to tumor cell invasion and metastasis in an LNCaP tumor progression model. The LNCaP-derived cell line series (LNCaP, C4, C4-2, C4-2B) were derived from serial passage through nude mice (Thalmann et al., 2000; Wu et al., 1994). The androgen sensitive parental LNCaP line is incapable of forming tumors in nude mice without stromal cell support. The C4 subline will form tumors when injected into castrated males, indicating that it is androgen insensitive, but will not metastasize. C4-2 is an androgen insensitive line that will metastasize, and the

C4-2B subline is an androgen insensitive line that rapidly forms bone metastases. When *PERLECAN* expression was assayed in the LNCaP series (Figure 3.5A) *PERLECAN* RNA and protein was present in all the prostate cancer cell lines at levels lower than the androgen sensitive LNCaP cells. Thus all the androgen insensitive prostate cancer cell lines expressed lower levels of PERLECAN RNA than he androgen sensitive cell line.

Inhibition of PERLECAN decreases prostate cancer cell proliferation in androgen sensitive and androgen insensitive tumor cells. To examine the direct effect of PERLECAN on cancer cell growth we examined the ability of small interference RNA (siRNA) directed at *PERLECAN* message to inhibit cell growth in the increasingly metastatic LNCaP cell line series LNCaP, C4, C4-2 and C4-2B. Proliferation assays demonstrated approximately equal decreases in BrdU incorporation for each cell line (Figure 3.5B). To evaluate the relationship between PERLECAN and androgens on cancer cell growth we performed BrdU incorporation studies on the androgen sensitive LNCaP cells utilizing androgen blockade with bicalutimide (Casodex) with *PERLECAN* siRNA or a scrambled siRNA control (Figure 3.5C). Independent application of *PERLECAN* siRNA or androgen blockade resulted in 28% and 45% decreases in BrdU incorporation respectively. When combined, PERLECAN siRNA and androgen blockade resulted in an additive effect with a 62% reduction.

PERLECAN correlates with SHH expression. Since androgen signaling and PERLECAN effects on tumor cell proliferation are independent, we asked what other signaling pathway PERLECAN might be modulating to support prostate cancer cell growth. Others and we have recently shown that SHH regulates prostate cancer cell growth (Datta and Datta, 2006; Fan et al., 2004; Karhadkar et al., 2004; Sanchez et al., 2004; Sheng et al., 2004). Since PERLECAN has been implicated in Hedgehog signaling in *Drosophila* (Park et al., 2003), we examined the correlation and interaction of PERLECAN with SHH in prostate cancer samples.

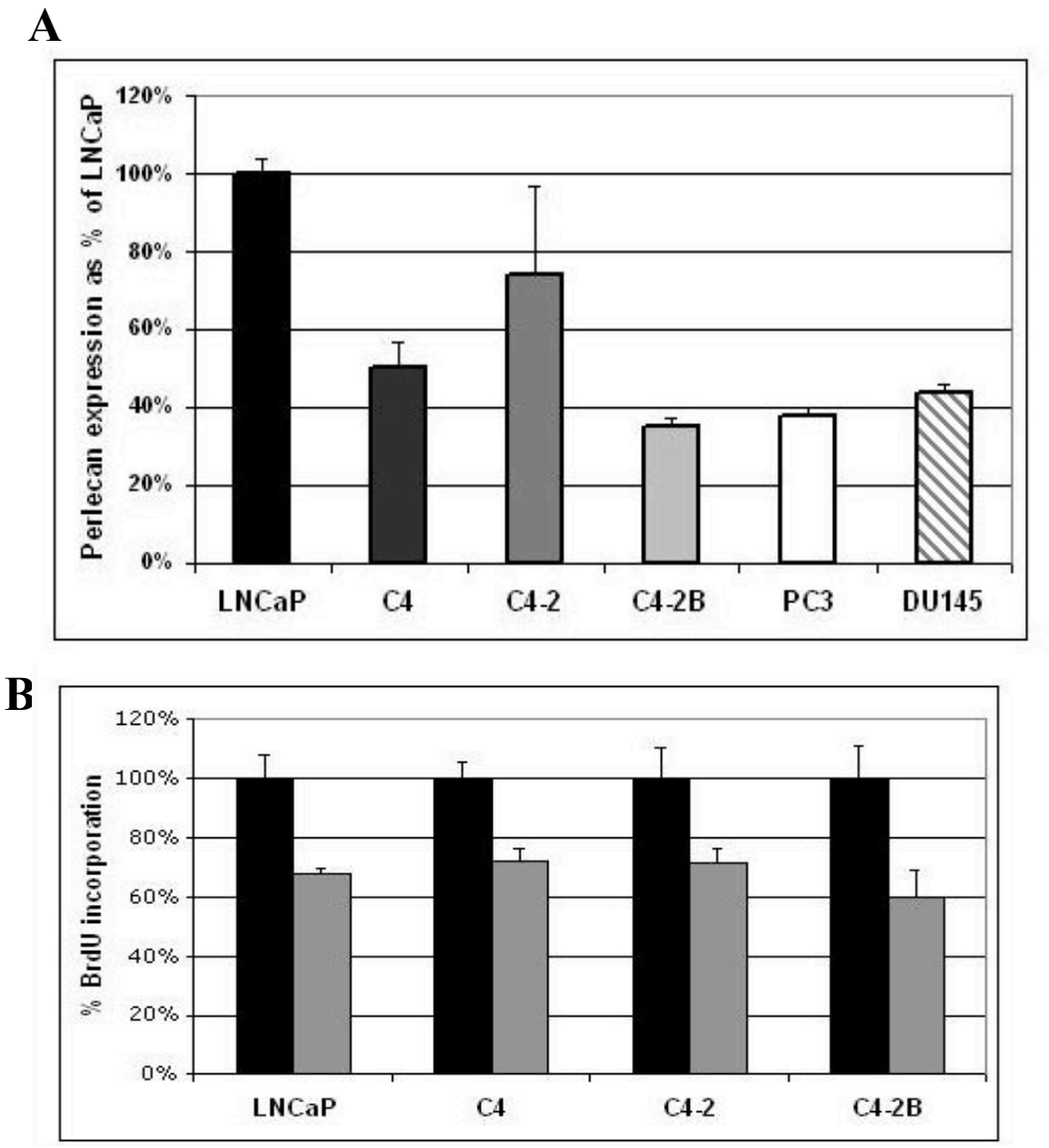


Fig. 3.5. PERLECAN expression and functional analysis in cell lines.(A) Relative PERLECAN mRNA levels from Realtime PCR (LNCaP series) and spotted cDNA microarray data (LNCaP, DU145, PC3). All samples presented normalized to LNCaP at 100%. Androgen sensitive: LNCaP. Androgen insensitive: C4, C4-2, C4-2B, PC3, DU145.(B) Inhibition by PERLECAN siRNA decreases prostate cancer cell proliferation. BrdU incorporation in the LNCaP, C4, C4-2 and C4-2B cell lines. All samples were normalized to control (scrambled siRNA treated) cells at 100%. Black bars represent control samples transfected with scrambled siRNA. Grey bars represent samples transfected with PERLECAN siRNA. Error bars represent n = 3 independent samples.

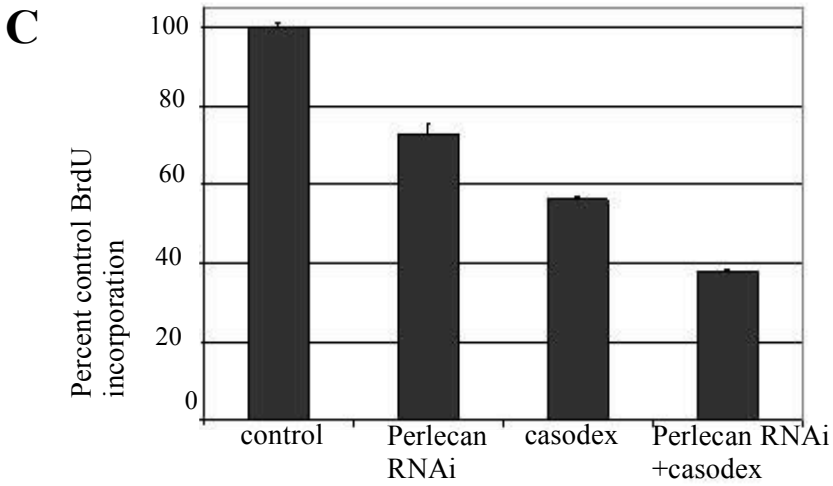
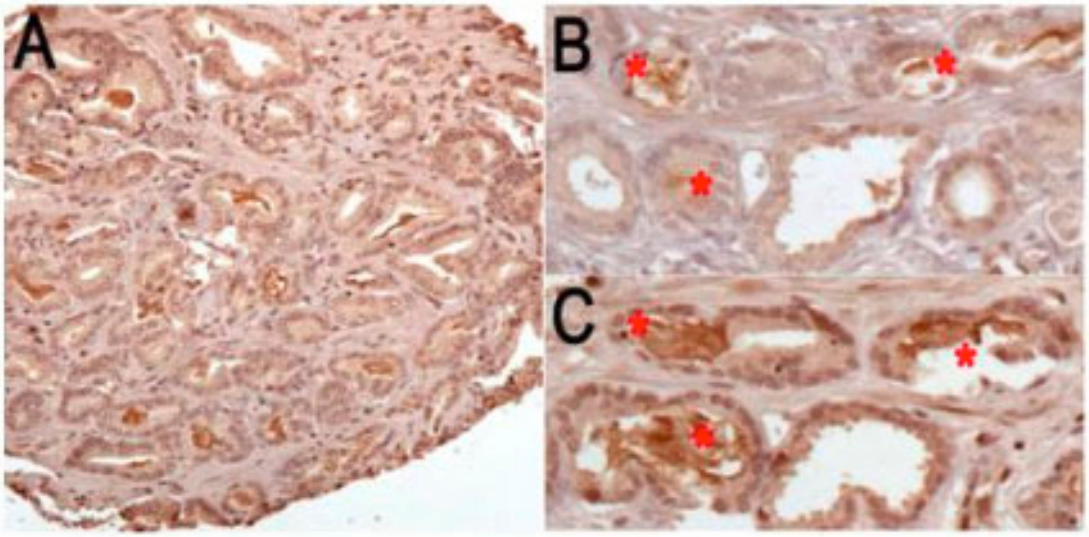


Fig.3.5. Continued. (C) Additive effect of PERLECAN siRNA and androgen blockade on cell proliferation. BrdU incorporation in LNCaP cells after PERLECAN siRNA and/or bicalutimide (Casodex) treatment. Control and Casodex alone samples were treated with a scrambled siRNA. $p < 0.0001$ for comparisons between groups. Error bars represent $n = 6$ for independent transfections.

Using sequential slides from tissue microarrays we compared the staining patterns for PERLECAN and SHH (Figures 3.6 A–C). Colocalization of PERLECAN and SHH staining was noted in a significant number of tumors, while luminal SHH was not observed in normal prostate controls. In addition, colocalization of both PERLECAN and SHH correlated with increased tumor cell proliferation as shown by Ki-67 (PCNA) staining (Figure 3.4D). Our previous studies (Sanchez et al., 2004) had examined expression of SHH pathway genes in six matched benign and tumor patient samples where we have also examined PERLECAN mRNA or protein expression (Figure 3.6G, 3.6H). In four common samples where we observe up-regulation of PERLECAN in tumor tissue, we previously detected up-regulation of *SHH*, *PTCH1* and *GLI1* (patients 945, 1854, 921 and 1866) suggesting a complete functional pathway in these tumors. In two common samples where we observe decreased *PERLECAN* mRNA levels, we previously saw decreased *SHH* expression (patients 829 and 887). Thus in individual patients, tumor expression of *PERLECAN* and *SHH* are correlated, in agreement with the colocalization of PERLECAN and SHH in tissue microarrays.

Inhibition of PERLECAN blocks SONIC HEDGEHOG signaling in cancer cells. To investigate whether PERLECAN is directly involved in modulating SHH signaling we examined the effect of *PERLECAN* siRNA on expression of *PTCH1* and *GLI1,* transcriptional targets of the SHH-GLI pathway (Lee et al., 1997) in LNCaP cells. Real-Time PCR analysis of *PERLECAN* siRNA treated cells revealed the expected 80% decrease in PERLECAN RNA, along with an 80% decrease in the level of *PTCH1* expression and a 90% decrease in *GLI1*expression compared to controls (Figure 3.7A). A similar decrease in PERLECAN protein levels in PERLECAN siRNA treated LNCaP cells compared to control siRNA was noted (data not shown). These results demonstrate that PERLECAN is required in androgen sensitive prostate cancer cells to achieve maximal SHH signaling activity. Given that PERLECAN has been shown to modulate the signaling of multiple growth factors including FGF2, FGF10 and VEGF, we asked if



D

Sample Co-localization	number of samples	mean % of Ki-67 positivity	Two-tailed ANOVA
PERLECAN and SHH positive	58	7.620690	p=0.0376
PERLECAN and SHH negative	270	5.283582	

Fig.3.6 Colocalization of SHH and PERLECAN, and correlation with Ki-67 staining. Immunohistochemistry for Sonic Hedgehog (A), demonstrating both weak cytoplasmic staining in prostate cancer epithelial cells and stronger intraluminal staining of secreted SHH. Colocalization of PERLECAN (B) and Sonic Hedgehog (C) in consecutive sections of prostate carcinoma. Examples of colocalization of the secreted proteins in gland lumens are highlighted (red asterisks). All histologic images originally photographed at 400 X magnification. Significant colocalization of PERLECAN and SHH staining was associated with higher cellular proliferation rates as indicated by Ki-67 nuclear staining by immunohistochemistry (D).

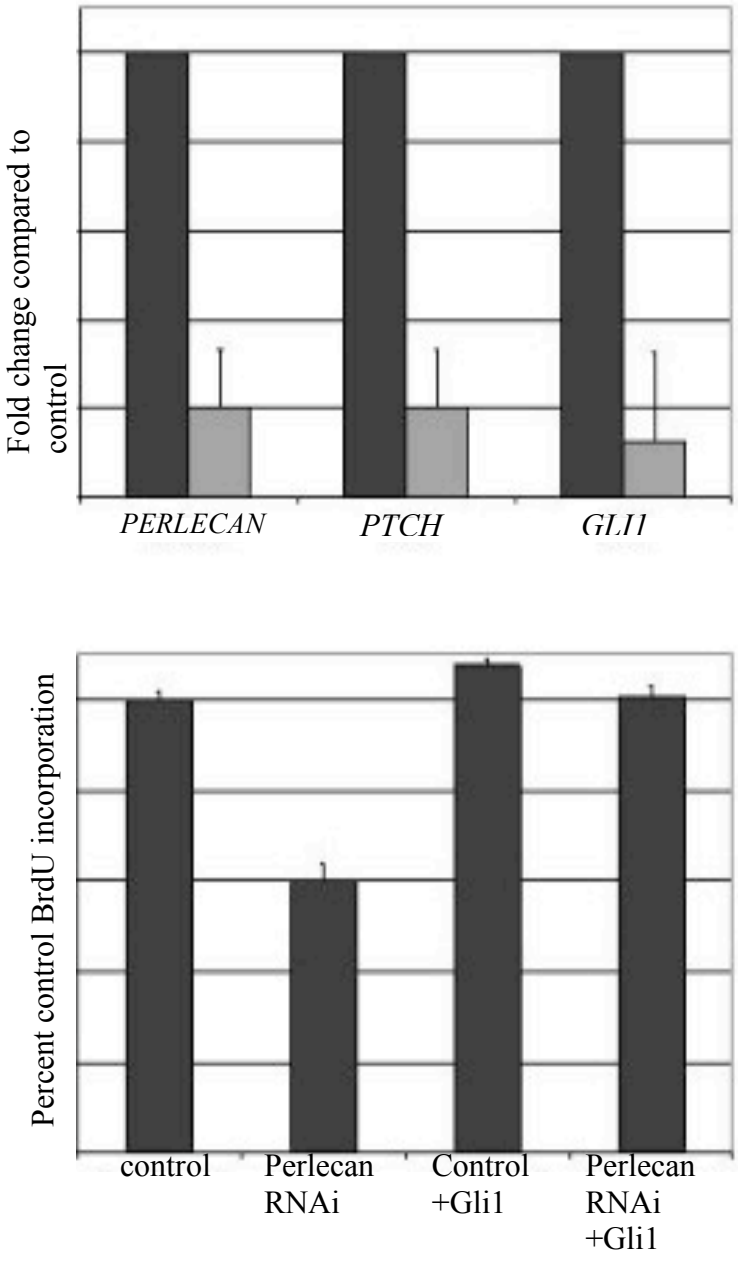


Fig.3.7. PERLECAN and the SHH-GLI1 pathway. (A) Decreased PERLECAN and SHH signaling in *PERLECAN* RNAi treated LNCaP cells. Expression of *PERLECAN*, and the SHH signaling molecules *PTCH1* and *GLI1* as determined by Real Time PCR. Black columns represent control samples, Grey columns represent PERLECAN RNAi treated cells. All expression normalized to β-*actin* levels. Real Time PCR studies were run with an n = 9. Error bars indicate standard deviation. (B) Gli-1 transfection restores BrdU Proliferation in PERLECAN RNAi treated cells. Percent BrdU incorporation normalized to levels of BrdU incorporation in control (scrambled RNAi treated) cells. BrdU analysis was done with n = 6. Error bars indicate standard deviation.

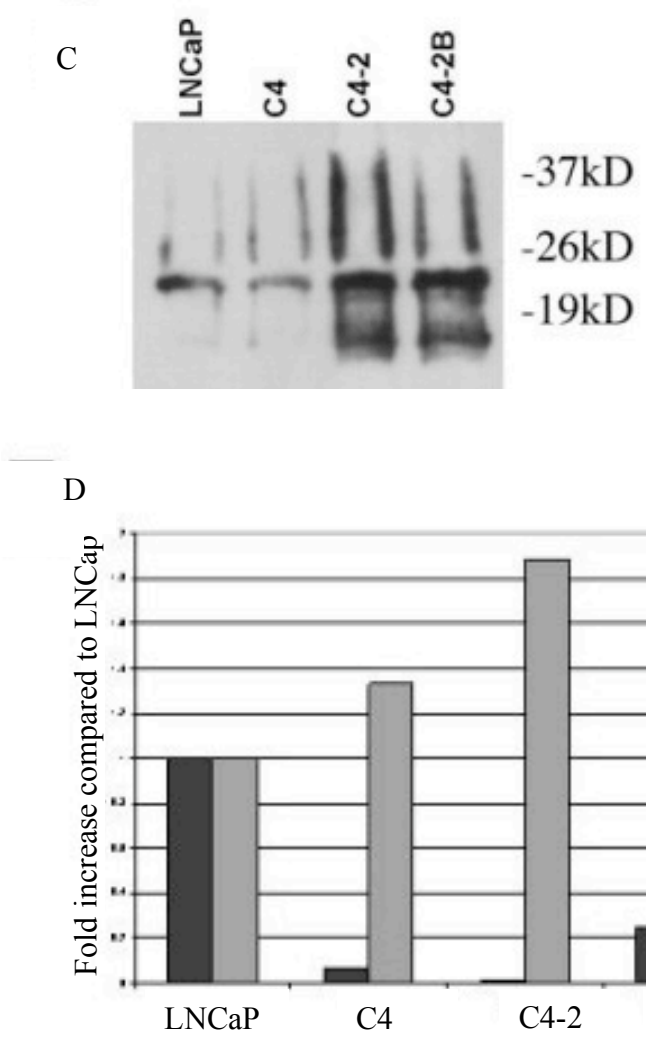


Fig.3.7. Continued. (C) Immunoprecipitation with anti-PERLECAN antibody pulls down SHH. Co-immunoprecipitation of SHH and PERLECAN from equal amounts of medium conditioned by 80% confluent cells. Size marker is indicated. Due to modifications, mature SHH runs as an approximately 22 kD band. Note the increased amount of bound SHH in the C4-2 and C4-2B cell lines. (D) Relative expression of the SHH pathway components in LNCaP series cells. Black columns represent SHH mRNA, grey columns represent PTCH mRNA, with expression presented as ratios with respect to expression in LNCaP cells. While *SHH* is lower, *PTCH* is higher in the androgen insensitive metastatic cell lines C4-2 and C4-2B compared to LNCaP. All mRNAs by QRT-PCR were normalized to Beta-actin.

the reduction of prostate cancer cell growth in PERLECAN siRNA treated cells was a result of decreased SHH signaling. If the decreased BrdU incorporation was due to inhibition of SHH signaling, then expression of the SHH downstream effector *GLI1* should rescue the effects of PERLECAN siRNA treatment. LNCaP cells were simultaneously transfected with PERLECAN siRNA and an expression vector for *GLI1* and their proliferation compared to that of controls transfected only with PERLECAN siRNA (Figure 3.7B). As we observed earlier, transfection of PERLECAN siRNA alone resulted in a drop in BrdU incorporation compared to controls. When PERLECAN RNAi and the GLI1 expression vector were co-transfected, the percentage of BrdU labeling returned to control levels. Transfection of the *GLI1* expression vector alone did not appreciably change LNCaP cell proliferation. This demonstrates that the major role of PERLECAN in LNCaP cells is to maintain levels of SHH signaling.

PERLECAN forms a complex with SHH. Finally, we asked how PERLECAN might affect signaling by SHH. Previously, we had demonstrated that PERLECAN from flies or mice forms a complex with Hedgehog(Park et al., 2003). To test for a tumor cell complex containing both PERLECAN and SHH we performed co immunoprecipitation studies from the LNCaP series (Figure 3.7C). PERLECAN-SHH complexes were detected in the conditioned medium of all cell lines under normal growth conditions. The mature SHH protein was identified by Western blotting in all protein extracts precipitated with anti-PERLECAN antibodies but not from extracts precipitated with control antibodies. Increased amounts of SHH-PERLECAN complexes were detected in C4-2 and C4-2B, the two metastatic cell lines. The level of PERLECAN protein does not change appreciably in the LNCaP series (Figure 3.8B), while the levels of *SHH* mRNA decrease across the series with increasing metastatic potential (Figure 3.7D). The presence of higher levels of SHH bound to PERLECAN in the C4-2 and C4-2B cells when the levels of PERLECAN protein are similar across the cell lines suggests increased binding of SHH to the available PERLECAN. The increased amount of bound SHH is apparently functional, as Real-Time PCR studies indicate a relative increase in

PTCH1 expression with respect to *SHH* in C4-2 and C4-2B when compared to LNCaP (Figure 3.7D). Taken together, the results of our expression, inhibition, and biochemical studies link *PERLECAN* expression and function to SHH-GLI pathway activity in advanced prostate cancer cells.

Tumor cells maintain PERLECAN under poor androgen/growth factor conditions. The LNCaP series showed a decrease in BrdU incorporation in response to PERLECAN siRNA, indicating PERLECAN based growth dependence under normal conditions regardless of their tumorigenic or metastatic potential. Our tissue microarray studies showed a correlation between PERLECAN/SHH colocalization and both higher Gleason grade and stronger Ki-67 staining, suggesting that more aggressive or metastatic cells are more likely to use PERLECAN-mediated SHH signaling. Since rapidly growing tumors tend to create microenvironments depleted of growth factors we asked if growth factor/androgen depletion via serum starvation would trigger the upregulation of *PERLECAN* in an effort to more effectively use limiting growth factors such as SHH. In the parental LNCaP cell line, PERLECAN mRNA levels decreased upon serum starvation (Figure 3.8A). Androgen insensitive C4, C4-2 and C4-2B lines maintained or increased their levels *PERLECAN* expression upon serum starvation. Immunoblotting for PERLECAN protein confirms these results under normal and serum starvation conditions (Figure 3.8B). We then asked if the expression of *PERLECAN* in more metastatic lines under poor growth conditions correlated with SHH signaling activity. Real-Time PCR analysis for mRNA expression of *SHH* and *GLI1* upon starvation (Figure 3.8C) demonstrated that expression of both genes increased in the more tumorigenic and metastatic cell lines. Thus the level of *GLI1* expression correlates with changes in *PERLECAN* expression upon serum starvation in the LNCaP series (Figure 3.8A). This suggests that tumor cells such as C4, C4- 2 and C4-2B that are capable of forming tumors and/or metastasizing without stromal support maintain a high level of SHH signaling under adverse growth conditions by maintaining high levels of *PERLECAN* and *SHH* expression.

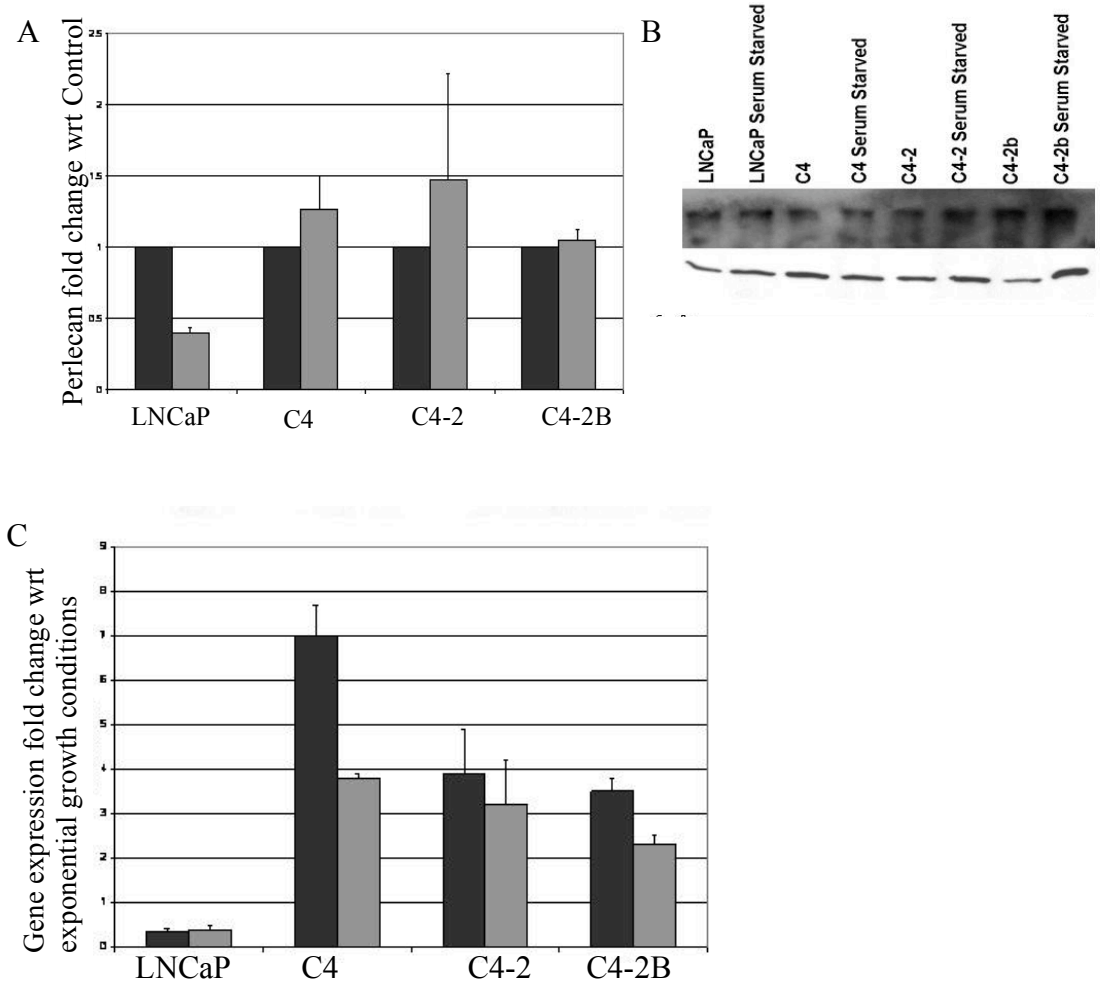


Fig.3.8. PERLECAN function under androgen and growth factor limitation. (A) Minimal changes in PERLECAN mRNA levels in LNCaP-derived cell lines upon serum starvation. RealTime PCR analysis of PERLECAN mRNA levels presented as fold increase in PERLECAN under normal (black bars) or starved (grey bars) growth conditions. While PERLECAN mRNA is decreased in LNCaP, all other cell lines demonstrate no change in PERLECAN mRNA levels.(B) Top Panel: No change in PERLECAN protein levels upon serum starvation. Agarose based western blots from protein extracts derived from exponentially growing or serum starved LNCaP, C4, C4-2, and C4-2B cells. No significant differences are noted in protein levels between the cell lines or under the differing conditions. Bottom Panel: Equivalent amounts of the same samples loaded on traditional SDS PAGE and probed for GAPDH as a loading control. C. Increases in expression of *SHH* and Gli-1 mRNA upon serum starvation. Real-Time PCR analysis of *SHH* (black bars) and *GLI1* (grey bars) as increased fold change compared to normal growth conditions. Gene expression determined by All Real Time PCR with an n = 9 and normalized to Beta-actin. Error bars indicate standard deviation.

Discussion

PERLECAN, a candidate oncogene for the CAPB locus. Using a bioinformatics based approach we identified PERLECAN as a candidate oncogene involved in both prostate cancer and glioblastoma multiforme based on its genetic association with the CAPB locus at 1p36. Here we demonstrate PERLECAN's expression and functional role in prostate cancer, and link it to the SHH pathway known to be involved in glial tumorigenesis (Dahmane et al., 2001). Thus from genetic mapping, physiological, and expression data there is evidence to suggest that PERLECAN is a strong candidate for the CAPB oncogene. The results of interference with PERLECAN function demonstrate that this proteoglycan is required for the growth of prostate cancer cells, extending its previously described roles in melanoma, colon, and lung cancer (Cohen et al., 1994; Nackaerts et al., 1997; Sharma et al., 1998) and emphasizing PERLECAN's role in multiple tumor types. Of note, genetic mapping studies have also identified a link between familial melanoma and 1p36, providing another link between PERLECAN and tumorigenesis (Greene, 1999).

PERLECAN's regulation of growth factors and the link to SONIC HEDGEHOG. As PERLECAN has been shown to bind a variety of growth factors in different tumors, the question as to which growth factor is being modulated in prostate cancer arose. Sonic Hedgehog has been associated with brain tumors and melanomas, two tumors with known genetic links to 1p36, where *PERLECAN* is located (Greene, 1999; Janer et al., 2003). Sonic Hedgehog has recently been linked to prostate cancer through a variety of studies (Datta and Datta, 2006). We have demonstrated an increased frequency of SHH positivity in prostate cancer tissue microarrays, and that SHH signaling regulates tumor cell growth in both primary prostate tumor samples and prostate cancer cell lines (Sanchez et al., 2004). High levels of SHH activity, as monitored by *PTCH1*, *GLI1* or *HIP* expression, are present in all metastatic prostate cancer samples that have been tested (Karhadkar et al., 2004; Sheng et al., 2004). In fact, high levels of *PTCH1* and

HIP expression correlate with high (8–10) Gleason scores (Sheng et al., 2004)where we have observed *PERLECAN* expression. Furthermore, activation of the SHH pathway by expression of *Gli* in the low metastatic potential rat AT2.1 cell line produced highly metastatic behavior, suggesting that high-level activation of the Sonic Hedgehog pathway determines metastatic behavior (Karhadkar et al., 2004). Finally, Sonic Hedgehog promotes the growth of LNCaP derived xenograft tumors in mice (Fan et al., 2004). We examined the potential of PERLECAN to regulate Sonic Hedgehog signaling in tumors. The importance of heparan sulfate proteoglycans for SHH signaling has been demonstrated in neural development, as mutations in the heparan sulfate binding site on SHH causes decreased SHH-driven proliferation (Rubin et al., 2002). In *Drosophila*, mutations in either PERLECAN, or heparan sulfate synthesis or modification genes, greatly perturb Hedgehog signaling efficiency by affecting Hedgehog transport and binding (Bellaiche et al., 1998; Bornemann et al., 2004; Datta, 1995; Datta et al., 2006b)]. Here we extend these findings in development to neoplasia by demonstrating that SHH both colocalizes and directly binds to PERLECAN in tumors, and that SHH signaling occurs through PERLECAN. This links PERLECAN to the SHH-Patched-Gli signaling pathway involved in prostate cancer (Datta and Datta, 2006), where PERLECAN acts to modulate the effects of SHH. As the SHH signaling pathway has been linked to multiple tumor types including prostate, stomach, brain, and skin tumors (Datta and Datta, 2006) this evidence suggests a more general role for PERLECAN in tumor regulation and tumorigenesis. We have surveyed a variety of tumor types and found SHH and PERLECAN colocalization in a number of these, such as squamous cell carcinomas and adenocarcinomas of various origins along with tumors deriving from areas of normal *PERLECAN* expression such as chondrosarcomas and osteosarcomas (data not shown).

PERLECAN in familial versus sporadic prostate cancers. We have demonstrated a positive correlation between PERLECAN immunostaining and prostate tumors, in particular for high Gleason score tumors (Table 3.4). While genetic mapping studies

make PERLECAN an excellent candidate for the CAPB oncogene, our clinical validation has been performed on prostate samples without information regarding their familial prostate cancer history. Due to the rarity of families with familial brain and prostate tumors, it is most likely that the tumors studied do not represent CAPB kindreds. The suggested role of PERLECAN in up-regulating SHH signaling in sporadic prostate tumors, combined with its association with a prostate cancer genetic susceptibility locus, places PERLECAN among a small group of genes with links to both familial and sporadic prostate cancers. This dual placement implies that PERLECAN is part of a common oncogenesis pathway that both familial and sporadic tumors may traverse during oncogenesis. Of note, other members of the SHH pathway, na namely *SU(FU)*, *GLI1* and *SMOH* also map to areas implicated in familial genetic studies (Datta and Datta, 2006)and are up-regulated in studies of sporadic prostate cancer tumors (Karhadkar et al., 2004; Sanchez et al., 2004; Sheng et al., 2004). Thus combining genetic analyses with evaluation of spontaneous tumors may allow us to identify the common pathways for carcinogenesis.

PERLECAN's role in prostate tumor growth: selective growth advantage for aggressive tumor cells under low androgen and/or growth factor conditions. High levels of PERLECAN protein correlate significantly with aggressive, highly proliferating prostate tumors in our tissue microarrays and are also up-regulated in aggressive tumors from individual patients. Yet PERLECAN is not present or overexpressed in every tumor or even in every metastatic site of tumor spread. While this result is not surprising considering the heterogeneity of neoplasia, it does suggest that subsets of tumors may utilize PERLECAN signaling in specific situations. This correlation is demonstrated in the varied responses of the LNCaP-derived prostate cancer cell lines under poor growth conditions. In these situations *PERLECAN* expression is maintained in the C4, C4-2, and C4-2B cell lines capable of forming stromaindependent tumors while the LNCaP parental line requires stromal support to form tumors and cannot maintain the PERLECAN specific growth advantage (Wu et al., 1994). This trait suggests a survival

benefit to the more tumorigenic and metastatic tumor cells. Under poor growth conditions where low androgen and growth factor concentrations are present, the increased presence of PERLECAN and its ability to concentrate growth factors would provide a survival advantage for tumor cells until a more suitable microenvironment can be found. In fact, our studies show that relative up-regulation of PERLECAN expression by the more metastatic lines during serum starvation allowed them to maintain their levels of SHH stimulation, while the relative down-regulation of PERLECAN expression in LNCaP resulted in decreased SHH signaling activity. Even under normal growth conditions, the more metastatic cell lines were able to form more PERLECAN-SHH complexes and obtain greater SHH stimulation. Thus in the changing tumor microenvironment the more metastatic tumor cells have a choice of pathways (androgen, PERLECAN-SHH) that can be modified or modulated to maintain tumor growth. Heparan sulfate proteoglycans such as PERLECAN have been shown to bind growth factors and may act as reservoirs or co-receptors for many growth factors (Wu et al., 1994). Thus increasing PERLECAN levels under growth factor limiting conditions such as within an inadequately vascularized tumor would be beneficial to a tumor cell. We propose that PERLECAN may sustain the growth of nutrient starved prostate cancer cells in rapidly spreading tumors by amplifying their sensitivity and response to SHH signaling. These findings are summarized in a model of PERLECAN action (Figure 3.9); in microenvironments with decreased growth factors and androgen, such as those encountered by rapidly growing tumors, PERLECAN provides a secondary pathway for growth through SHH. This is used in both the androgen responsive and androgen insensitive aggressive tumor cells. Based on this model, one would hypothesize that chemotherapeutic treatments that simultaneously target both the androgen and the PERLECAN-mediated SHH pathways would provide the best control of

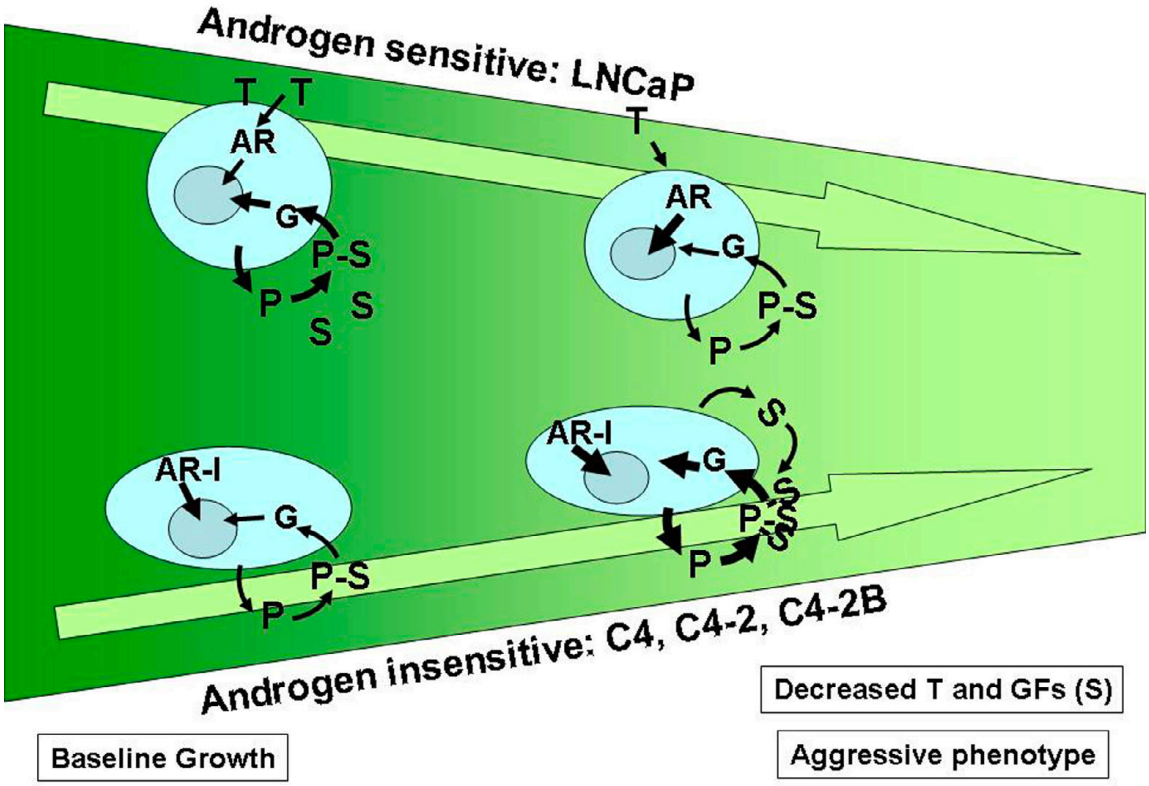


Figure 3.9. Modulation of androgen and PERLECAN regulated SHH signaling. As changes occur to the tumor microenvironment, prostate cancer cells modulate their use of both androgen and PERLECAN mediated SHH signaling. The use of androgen (T) occurs via the androgen receptor (AR). PERLECAN (P) is produced, binds SHH (S) and signals through the Gli (G) proteins. The heaviness of each arrow indicates relative signaling strength (gene expression, complex formation). Androgen sensitive cells (LNCaP) utilize both androgen and PERLECAN-SHH signaling under normal conditions, but decrease PERLECAN-SHH signaling under poor growth conditions. In contrast aggressive androgen insensitive cells (C4, C4-2, C4-2B) utilize both pathways, and upregulate the PERLECAN-SHH signaling under poor growth conditions. This may occur through increased SHH binding affinity to PERLECAN.

androgen sensitive aggressive prostate cancer.

PERLECAN as a global regulator of growth factor action. While we have demonstrated that SHH is critical to PERLECAN-dependent cancer cell growth, other growth factors may also be regulated through PERLECAN at different times or in different clinical stages. Recent results (Savore et al., 2005) suggest that PERLECAN may regulate the activity of different growth factors during metastasis to bone. Thus the true role of PERLECAN may not be regulating a single growth factor, but its ability to allow the tumor cell to adapt to differing tumor microenvironments by facilitating the signaling of different growth factors. If this is shown to be true, PERLECAN may be an excellent target for drug targeting, with tumor specific targeting achieved through the selective blocking of specific growth factor binding sites on PERLECAN.

PERLECAN function in metastasis, a role in the bony matrix. PERLECAN is secreted by tumor cells, but is also present in specific stromal microenvironments in the body. This may affect a tumor's propensity to spread to specific sites. We have shown here that prostate cancer maintains PERLECAN expression when it spreads to the lung or liver, but is less likely to do this in the soft tissue or lymph nodes. Maintaining or finding "PERLECAN rich" sites may explain the propensity of tumors to home to specific sites during metastatic spread. A specific example of a PERLECAN rich site would be the bone extracellular matrix, a major site for prostate cancer metastasis. In these sites PERLECAN plays a role in normal bone formation and regulation through the modulation of growth factors utilized by osteoblasts (Hassell et al., 2002; Hecht et al., 2002; van der Horst et al., 2003). Recent studies using the bone-targeted prostate cancer line C4-2B show that PERLECAN is required for development of metastases through the modulation of growth factors, and leads to efficient tumor growth and vascularization (Savore et al., 2005). Thus it appears that the presence of PERLECAN in

the bony matrix may help explain the tropism of prostate cancer to the bony matrix. Use of PERLECAN as a drug target may prove advantageous by blocking bone metastasis and its associated morbidity. Lastly, PERLECAN, as a secreted protein, may prove to be a useful biomarker for metastatic prostate cancer as well as a marker of either the risk or detection of tumor metastasis to bone since it can be easily detected in urine or serum samples, respectively.

Methods

Bioinformatics based analysis for candidate genes in the CAPB region. The 1p36 region, as defined by the chromosomal basepair data present in the human genome build 16 from the UCSC Genome Browser datasets, was searched for defined genes as identified in the NCBI LocusLink database. This search identified 5,108 expressed exons comprising 659 identified transcripts and 619 defined genes. Using text mining we searched a dataset of 3,737 prostate cancer genes as defined by co localization of the gene name based on a hand annotated list from LocusLink and the words "prostate cancer" in MEDLINE. From this dataset 14 genes in the 1p36 region had been described in prostate cancer studies. A second text-mining search we identified 15 genes in the CAPB region that also had been described in studies of the brain. None of the genes in the brain or prostate cancer text mining datasets were common. We then focused our examination on CAPB region genes with associated data in brain studies, and prostate and prostate cancer expression data from the Cancer Genome Anatomy Project (CGAP) along with cDNA microarray expression data generated in our laboratory for the prostate cancer cell lines LNCaP, DU-145, and PC3. A comparison of these datasets revealed three genes, EPHA2, HSGP2, and CAP2B, with data in both brain research studies and expression in the prostate cancer or the precancerous change high grade prostatic intraepithelial neoplasia. Of these three genes, HSPG2 also was contained within our prostate cancer cell line cDNA expression datasets, with increased levels of expression in the derived invasive sublines of PC3 when compared to a derived non-invasive

subline.

Prostate samples and tissue culture. LNCaP, PC3 and DU-145 cell lines were obtained from ATCC and grown under standard conditions. The LNCaP series LNCaP, C4, C4-2 and C4-2B were obtained from Dr. L. Chung. All primary prostate tumors were obtained by MWD using approved protocols with informed consent on the part of the subjects.

Real-time PCR on cell line RNA samples. Total RNA isolated from cell lines using Trizol and then further purified using the RiboPure kit (Ambion). Purified RNA was digested with DNAse (Invitrogen), and analyzed using the SYBER Green system according to manufacturers protocols (Applied Biosystems) on an ABI Prism 7700 machine. Each sample was run in triplicate at three different concentrations. Primers were designed using Primer Express software and are available upon request. Fold increase/decrease comparisons were calculated using the delta-delta Ct method.

Tissue microarray and immunohistochemistry. Upon institutional review board approval, a tissue microarray was prepared from 288 radical prostatectomy cases present at the Medical College of Wisconsin. A second tissue microarray was prepared from samples collected under approved protocols at the University of Pittsburgh Medical Center. 0.6 mm cores were arrayed and 5 um sections processed. Benign tissue, high-grade prostatic intraepithelial neoplasia, or invasive tumor tissue were identified by MWD or RD by high molecular weight cytokeratin staining (CK903 Ab, DAKO). A third tissue microarray was prepared from samples collected under approved protocols as part of the rapid autopsy program at the University of Michigan. For microarray samples, a common antigen retrieval procedure was carried out. Slides were processed for PERLECAN or SHH and developed with HRP conjugated secondary antibodies and DAB substrate. For a portion of the tissue microarray anonymous de-identified pathologic and outcomes data were available. Individual cores were examined as duplicates and staining correlated using Chi-squared, Fisher's Exact or two-tailed

ANOVA analyses.

Transfection and proliferation assays. Purified and desalted siRNAs were purchased from Ambion as a proprietary non-validated *PERLECAN* siRNA and a scrambled siRNA control. SiRNA and *GLI1* expression vector transfections were carried out with Lipofectamine 2000 (Invitrogen) as described by the manufacturer and effects measured after 72 hours. Casodex was used in cell cultures as described previously. Immunocytochemistry on cell lines was carried out using with anti-BrdU (Research Diagnostics or Becton-Dickinson) and HRP-conjugated secondary antibodies (Boehringer Mannheim) using standard techniques.

Protein extracts, Western blotting and immunoprecipitations. Normal and tumor tissue from the same patients were obtained as described below following approved protocols. Sections were assessed pathologically by a urologic pathologist (MWD) to determine areas of normal and tumor tissue. Samples were microdissected and total protein isolated. Proteins were also isolated from cultured medium from cell lines grown under normal or serum starved conditions. Proteins were run on a 1.6% agarose gel, blotted and probed for PERLECAN (Chemicon). Equal samples were loaded onto a standard SDS-PAGE gel, blotted and probed for GAPDH (Santa Cruz) as a loading control. Equal amounts of conditioned medium from equivalently confluent cell lines were immunoprecipitated with an anti- PERLECAN or unrelated control antibody, the resulting complex run on denaturing SDS-PAGE, and the presence of SHH verified by immunoblotting (Santa Cruz).

Contributions

My contribution to the work in (Sanchez et al, 2004) et al involved analysis of the SHH signaling pathway in prostate tumors and matched normal tissue samples taken from six patients. This included processing of RNA extracts from prostate tissue, Primer design

and development of Real-time qPCR (define) analysis, and Real-time qPCR in matched tumor and normal samples to determine the expression levels of *SHH, PTCH1*, and *GLI1/2/3/* (Table 2.) My data analysis shows that all tumor samples have increased expression levels of all pathway components compared to normal tissue from the same patient. These results suggest that pathway activation correlates positively with prostate tumors. There is some notable variability between tumor samples, which could be attributed to the known heterogeneity of prostate cancer

My contribution to the work of (Datta et al, 2006) involved analysis of the levels of *PERLECAN* expression and PERLECAN protein in matched normal and tumor samples. This included primer design, development of a qPCR assay, and data analysis for *PERLECAN* expression levels in normal and tumor samples. Protein analyses included Dot Blot of protein extracts from matched tumor and normal samples to assay PERLECAN levels (Fig.3.8). Results of these experiments showed that 4 out of the 6 matched samples that had previously shown an increase in SHH signaling also showed an increase in *PERLECAN* expression. This suggests that a subset of tumors upregulate *PERLECAN* expression. In addition, 2 other matched samples showed an increase in PERLECAN protein. Together, the results of these and other experiments show a significant positive correlation between prostate tumors and PERLECAN upregulation.

The positive correlation between tumors and PERLECAN and SHH signaling, as well as the colocalization of these two elements in prostate tumors gave rise to the hypothesis that PERLECAN may be regulating SHH signaling in prostate cancer. To test this, I evaluated the effects of PERLECAN on SHH signaling in the prostate cancer cell line LNCaP. This included qPCR of PERLECAN RNAi treated LNCaP cells for *PERLECAN*, *PTCH1* and *GLI1* expression levels. Evaluation of *PTCH1* and *GLI1* expression levels in PERLECAN knockdown cells shows a decrease in the expression of *PTCH1* and *GLI1* (80% and 90%) respectively with PERLECAN RNAi. PERLECAN RNAi also causes a decrease in cell proliferation of about 40%. These results show that

PERLECAN is necessary for SHH signaling and proliferation in LNCaP cells. PERLECAN upregulation in prostate tumors also correlated with the site of secondary metastasis: primary tumors with high levels of PERLECAN are associated with metastases to the lungs and liver rather than soft tissue or lymph nodes. This raises the possibility of differential PERLECAN expression in prostate cancer cells. To test this hypothesis, we used the LnCaP cell model of prostate cancer progression (Thalmann et al., 2000; Wu et al., 1994). Initially I evaluated the levels of PERLECAN expression and SHH in LnCaP, C4, C4-2, and C4-2B cell lines. My determination of PERLECAN expression and SHH signaling in the LNCaP series shows that the androgen-insensitive prostate cancer cell lines showed lower baseline levels of *PERLECAN* expression than the androgen-sensitive LNCaP cell line. This result confirms differential expression of *PERLECAN* in prostate cancer cell lines, and raises the possibility that different types of cancer cells may differ in their ability to utilize PERLECAN upregulation as a means to sustain SHH signaling. To test this hypothesis, I evaluated both *PERLECAN* expression levels and SHH signaling in the LNCaP cell line model under serum starvation conditions, which mimic the low levels of nutrients and growth factors found in a rapidly growing tumor. I observed no significant increase in *PERLECAN* expression or protein when the LNCaP series lines were subject to serum starvation. However, I did observe an increase in the levels of SHH signaling that correlates with the more aggressive cell lines in the model. These results raised the possibility that PERLECAN in the LNCaP cell line model is not acting in a dose-dependent manner, but rather that PERLECAN from more aggressive cell lines is better able to bind SHH and therefore facilitate SHH signaling. This hypothesis was confirmed by co-immnunoprecipitation studies that showed higher levels of SHH binding in PERLECAN from C4-2 and C4-2B cell lines compared with LNCaP and C4 cell lines. Altogether, these results point to a model in which prostate cancer cells with invasive or metastatic potential are able to maintain growth in low androgen conditions using alterations in PERLECAN to sustain SHH signaling–dependent proliferation.

Alternate mechanism for *PERLECAN* regulation of SHH signaling in advanced prostate cancer

My published research, along with our collaborators' studies, has shown that SHH signaling is required for cell proliferation in advanced prostate cancer. Also my studies in cell lines have shown that PERLECAN regulates SHH–dependent proliferation and suggests a mechanism by which prostate cancer cells can switch from androgen signaling to SHH signaling in order to support growth under low androgen conditions. This mechanism includes changes in PERLECAN structure or composition that allow for better binding of the SHH ligand in order to support stronger SHH signaling. However, my data on matched tumor and normal samples from the same patients, as well as the tissue microarray data suggest that there is upregulation of *PERLECAN* expression and protein in a subset of prostate tumors. This would suggest an alternate mechanism of PERLECAN function in SHH signaling, where PERLECAN acts in a dose-dependent manner to control SHH signaling. In order to further evaluate this hypothesis, we used the PC3 prostate cancer cell line model (Kaighn et al., 1978; Pettaway et al., 1996). Here, I show that cells with invasive or metastatic potential are able to upregulate their levels of *PERLECAN* expression in starvation conditions that mimic the environment of a rapidly growing tumor. We also observe an increase in the amount of SHH signaling in a cell line with metastatic potential. Together, these results show that upregulation of PERLECAN levels is a second mechanism of increasing SHH signaling under stress conditions.

Materials and methods

Cell lines and prostate tissue. The PC3-NI, PC3-I, Pro4 and LN4 cell lines (Pettaway et al., 1996) were obtained from Drs. Balla, Lindholm and Pettaway, and grown as specified. Normal and tumor prostate tissues from the same patients were microdissected

from sections with a laser capture microscope after pathological assessment. All primary tumors were obtained by Dr. M.Datta using approved protocols with informed consent by the subjects.

RNA isolation and qRT-PCR. RNA was isolated using the Trizol reagent (Invitrogen), treated with DNAseI (Invitrogen), and reverse transcribed using the TaqMan Reverse transcription (Applied Biosystems) kit, using oligodT primers as described by the manufacturer. qPCR reactions were run on an ABI PRISM 7700 using SYBR green Master mix (Applied Bioystems). Primer sequences were designed using Primer Express software (Applied Biosystems) and are available upon request.

Results

PERLECAN is upregulated in cell lines with invasive and metastatic potential. We observed an increase in *PERLECAN* expression and protein in a subset of matched tumor samples compared to normal prostate. In addition, our tissue microarray data shows that *PERLECAN* upregulation correlates positively with higher proliferation levels and a higher Gleason Grade. This raises the possibility that cancer cells with aggressive behavior are able to upregulate PERLECAN. We hypothesize that more aggressive types of prostate cancer cells might have higher levels of *PERLECAN* expression compared to less aggressive cells. To investigate this hypothesis, we used two PC3 cell line models (Lindholm et al., 2000; Pettaway et al., 1996). The metastatic parental PC3 cell line was used for in vitro selection in the matrigel assay to yield the PC3-I line. In vivo selection using serial passage through nude mice resulted in the Pro4 line, with no metastatic potential, and the LN4 line, which can metastasize to the lymph node.

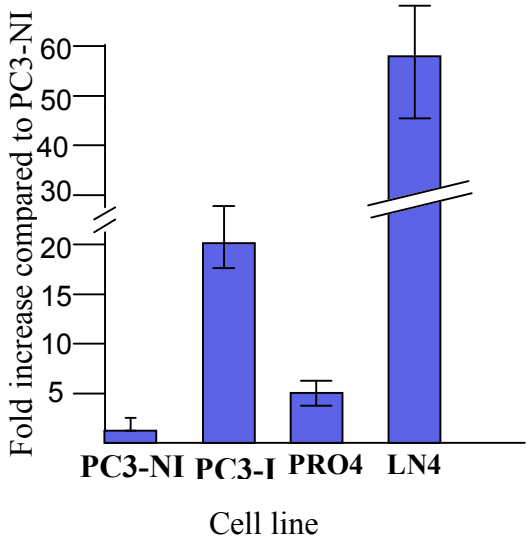
Earlier studies by our collaborator M. Datta (Schlicht et al., 2004) have shown that in a cDNA microarray study shows that PC3-I cells showed a 1.43 fold increase in *PERLECAN* expression with respect from PC3 NI cells, and LN4 showed a 1.40 fold

increase in *PERLECAN* levels compared to Pro4. We further evaluated *PERLECAN* expression levels in confluent cells using qRT-PCR. Fig 3.10 A shows that both the PC3-I line and the LN4 line have a higher *PERLECAN* expression than the PC3-NI and the Pro4 line. This suggests that cells with invasive or metastatic potential express higher baseline levels of *PERLECAN*.

Rapidly growing tumors can create low levels of growth factor conditions. Invasive and metastatic cells have to be able to utilize very efficiently the low levels of growth factors in order to be able to proliferate and grow in this type of environment. We used serum starvation to evaluate if there are any changes in *PERLECAN* expression upon stress conditions, and evaluated *PERLECAN* expression levels in starved cells. Figure 3.10 B shows that the PC3-I and the LN4 lines show a dramatic increase in their *PERLECAN* expression compared to the PC3-NI and the Pro4 lines under starvation conditions. If PERLECAN acts in a dose-dependent manner to upregulate SHH signaling in aggressive prostate cancer, we would expect an increase in SHH signaling under starvation conditions in the more aggressive cell lines. Fig 3.11 shows an increase in the expression levels of SHH and its response gene PTCH1 in LN4 compared to Pro4 levels. Altogether, these results suggest that PERLECAN acts in a dose-dependent manner to regulate SHH signaling in the PC3 series of prostate cancer cells.

I have shown a second mechanism for PERLECAN regulation of growth factor signaling. This mechanism relies on upregulation of *PERLECAN* expression under stress conditions, which enables cells to better respond to growth factor signaling. I have also observed a concomitant upregulation of SHH in the metastatic LN4 cell lines, in contrast with non-invasive, non-metastatic cell lines of the same origin. This suggests that *PERLECAN* upregulation enables stronger SHH signaling in a dose-dependent manner.

A



B

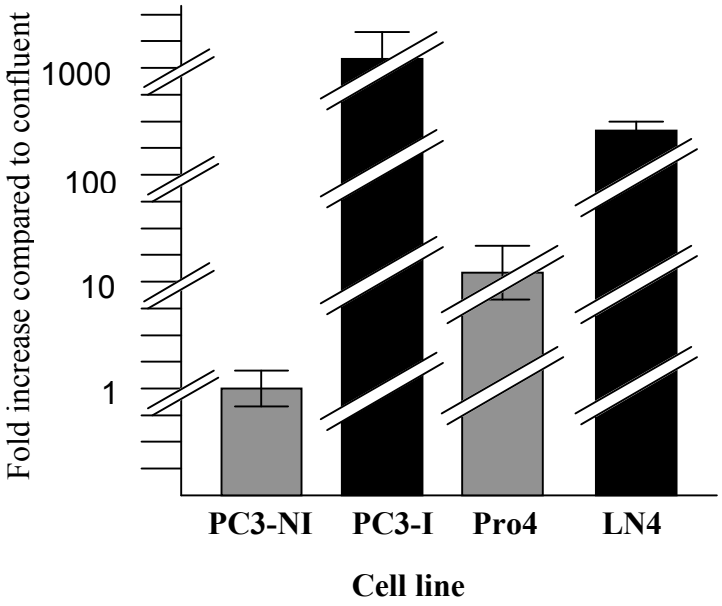


Fig. 3.10. Invasive and metastatic cell lines have increased *PERLECAN* expression. (A) Baseline PERLECAN expression. (B) PERLECAN expression in starved culture conditions compared to normal growth conditions.

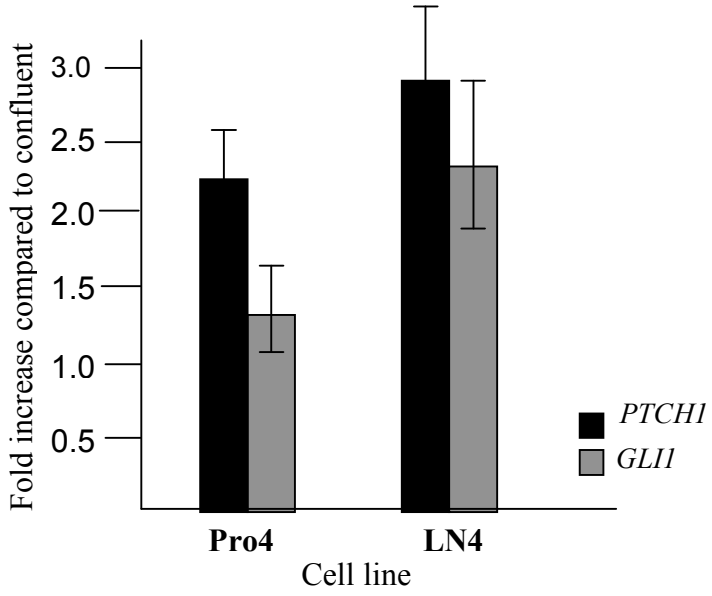


Fig. 3.11. Metastatic cell lines have increased SHH signaling**.** qPCR showing response gene expression in Pro4 and LN4 cell lines under starved conditions.

Fig. 3.12 shows a model of the proposed mechanism. Together with our previously published results, this work highlights the important role of PERLECAN, a heparan sulfate proteoglycan in the extracellular matrix, in regulating SHH signaling in advanced prostate cancer.

Discussion

Prostate cancer is one of the most clinically relevant significant neoplasias, and attempts to describe significant biomarkers and mechanisms of progression have been obscured by the heterogeneous nature of the disease. We have shown that upregulation of *PERLECAN* expression and SHH signaling correlates with tumors. Based on this positive correlation, we have manipulated the system using primary cultures and prostate cancer cell lines, and have revealed that prostate cancer proliferation can be inhibited by blockage of SHH signaling. This blockage can be achieved by inhibition of the SHH pathway at the level of Smoothened (as is the case with cyclopamine), at the level of the SHH ligand itself (with SHH blocking antibodies), and by altering the extracellular microenvironment (with PERLECAN RNAi). Although SHH signaling has been shown to play a role in other types of cancer, ours was the first report that directly implicated SHH signaling in advanced prostate cancer proliferation. Our results highlight the importance of the deregulation of developmental signaling in neoplasia. Other studies have addressed the role of SHH in prostate cancer metastasis, and found that it plays a crucial role in this process (Karhadkar et al., 2004; Sheng et al., 2004) while others have established a strong correlation between higher Gleason Grade prostate cancer and mutations in components of the SHH signaling pathway (Sheng et al., 2004). There have been a variety of mechanisms proposed for the deregulation of SHH in prostate cancer. It has been observed that SHH is produced in epithelial cells but is active in the underlying mesenchyme during normal prostate development. In other systems, such as

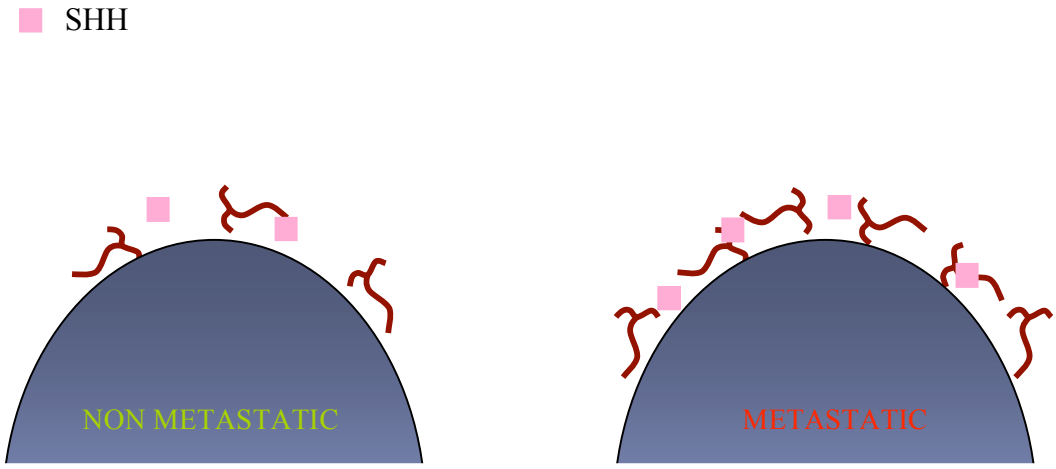


Fig. 3.12. Dose-dependent action of PERLECAN on SHH signaling. Under low growth factor conditions, PERLECAN allows for greater binding of SHH in a dose-dependent manner.

signaling. However, recent reports (Zhang et al., 2007) have shown that some prostate cancer cell lines are not able to respond to SHH signaling present in the media. conflicting reports may be due to different sensitivity between qPCR assays versus the GLI luciferase assay used in (Zhang et al., 2007). However, the possibility of differential response to SHH signaling gives rise to the question of whether SHH signaling activation is context dependent. In this case, the microenvironment will play a fundamental role in the deregulation of SHH signaling, and possibly other signaling pathways as well. In developmental processes. The ability of SHH to signal as well as the range of activity have been shown to be dependent on heparan sulfate proteoglycan function (Nybakken and Perrimon, 2002b). Here, we extend these findings into the field of cancer progression, where we show that PERLECAN regulates SHH signaling in a number of prostate cancer cell lines, and it has a significant effect on proliferation in LNCaP cells. We also show that more aggressive cell lines are able to either upregulate levels of PERLECAN, or produce PERLECAN with different SHH binding abilities. Given that PERLECAN upregulation correlates significantly with highly proliferative prostate tumors, and that it is colocalized with SHH in the prostate, PERLECAN is likely to be necessary for SHH to stimulate proliferation of advanced prostate cancer. Furthermore, because PERLECAN has also been shown to regulate signaling by a number of different growth factors, including FGF-2 and VEGF-A (Savore et al., 2005), It may act as a regulating center in the microenvironment, and stimulate cancer cell growth by modulating signaling by different growth factors. This makes PERLECAN an atracive drug target for treatment strategies. Furthermore, the correlation between PERLECAN and high levels of proliferation in advanced prostate cancer makes it a possible biomarker in preventive strategies.

The initial characterization of PERLECAN requirement for full-strength SHH signaling was initially described in the model organism *Drosophila* (Park et al., 2003). Conservation of this mechanism of SHH regulation from flies to human prostate cancer highlights the importance of research in lower organisms as relevant in human disease.

This raises the possibility that the fruit fly *Drosophila* could be a productive model in which to formulate and test initial hypotheses about other risk factors affecting prostate cancer progression. In the next chapter, I will explore the feasibility of a prostate cancer and prostate aging model in *Drosophila*.

CHAPTER IV
THE *DROSOPHILA* EJACULATORY BULB AS A MODEL FOR PROSTATE CANCER AND PROSTATE AGING

Introduction

Prostate cancer is a complex heterogeneous multifactorial disease. Most deaths from prostate cancer are due to advanced prostate cancer, a form of the disease that is highly invasive, metastatic, and androgen–insensitive (Feldman and Feldman, 2001). Of the risk factors for prostate cancer, age is the most important: according to the latestdata from the SEER registry, almost 2 thirds of prostate cancer occurs in men over 65 years of age (SEER registry Database, 2008). There are also reports of old age correlating with a more aggressive phenotype (Alexander et al., 1989). Some attempts at explaining the correlation between aging and aggressive cancer include difficulties with diagnosing early stages of disease in older patients, which will cause a bias towards diagnosis at a later stage. An alternate explanation involves the fact that it takes a long time to accumulate the set of mutations in oncogenes and tumor suppressors that will lead to the disease. However, emerging research in both the aging and cancer fields suggests that there are aspects of the aging process that actively influence tumorigenesis and cancer progression. Apart from evidence that aging influences initial steps in tumorigenesis, such as genomic instability and telomere integrity (Blasco, 2007), reports from the Campisi lab describe the secretion of numerous growth promoting factors such as FGF into the extracellular matrix by senescent fibroblasts (Krtolica et al., 2001). Taken together with the fact that cancer cells behave differently in young compared to older hosts (Hirayama et al., 1993; McCullough et al., 1997) we can see that aging has a crucial impact on cancer progression, both at the cellular and microenvironment levels.

Until very recently, aging was simply thought of as the progressive breakdown of the systems that maintain cell and tissue homeostasis, resulting in a progressive decay in normal cellular and tissue function. However, emerging research has shown that there are a number of signaling pathways and conditions that influence the rate and onset of aging. One of the first fundamental observations was the fact that calorie restriction led to an increase in lifespan that was conserved in evolution (Haigis and Guarente, 2006). In organisms like yeast or nematodes, extreme calorie restriction was accompanied by a quiescent state, such as sporulation or diapause, in which metabolism was reduced and reproduction was prevented. However, when elucidating the molecular mechanisms that underline responses to calorie restriction, it was found that longevity could be uncoupled from diapause. This revealed the first molecular insights into the nature of longevity, which highlighted the important role of histone deacetylaces (such as Sir2) and Foxo transcription factors (such as DAF-16) (Haigis and Guarente, 2006) (Kenyon, 2001). Further work revealed that for the most part, calorie restriction works by inhibiting the Insulin/Insulin-like growth factor (IGF) pathway, which in turn elicits a response that involves the nuclear recruitment of the Foxo transcription factors and results in increased longevity. Elements of this pathway are conserved from nematodes and *Drosophila* to mammalian models. *Drosophila* has lent itself to the study of aging in part because of its short lifespan (80 days), and also because of the availability of long-lived mutants, such as *indy* or *chico* (Helfand and Rogina, 2003). Work on these systems has revealed that both of these mutations compromise the fly's metabolism in a way that mimics calorie restriction. Recent work from the Pletcher lab (Libert et al, 2007) has revealed that there is a neurological component to the increased longevity of calorie restriction: just the perception of food by olfactory receptors, even in the absence of uptake, will have a significant impact on lifespan.

Parallel with organismal aging, studies have been undertaken to better understand the mechanisms of cellular aging, as well as the physiological behaviors of the aging cell. One process that has attracted a great deal of attention and research is cellular

senescence, which is a state of arrested growth and altered behavior which correlates with aging, although it can be observed in response to stress conditions. The role of senescent cells in altering the tissue microenvironment and stimulating cell proliferation is documented (Nelson and Bissell, 2006), although little is known about the effects of senescent cells *in vivo*. Currently, it is thought that senescent cells present in the stroma of various organs can contribute to the changes in microenvironment that stimulate the growth of precancerous lesions. Current approaches to understanding the invasive/metastatic process take into account the impact of the microenvironment, both in terms of extracellular signaling molecules secreted by cancer cells, and ECM proteoglycans that aid in the transport and signaling process. It is now clear that aging has an impact on these processes that will therefore influence the progression of the disease.

Research on the aging microenvironment and its impact on cancer has been undertaken by two different approaches: the first involves culturing cells *in vitro*, the second implantation of cells in senescent hosts. In the first approach, cells are cultured with media from senescent cells, or co-cultured with senescent fibroblasts. However, as the effects and significance of senescent cells in vivo have not been well documented, there may be other factors in the microenvironment that this model cannot recapitulate. The second approach involves implantation of cancer cells into host mice or rat models, and evaluation of the age-dependent change in the rate of tumorigenesis/ metastasis. This approach, albeit useful, is costly and time-consuming, and the average lifespan of mouse and rat models, in the order of years, is especially cumbersome for aging studies. A simple model system such as *Drosophila* will be a strong tool in the study of the aging microenvironment and its role in cancer. It provides a simple organism in which changes in microenvironment with age can be modeled and their impact on cancer progression can then be evaluated,

Drosophila is an established model in aging studies. The short lifespan of the fruit fly (80 days) and the vast array of genetic and molecular tools available to the *Drosophila* biologist enable a variety of manipulations in vivo to address aging. In addition *Drosophila* provides specific aging interventions such as temperature shifts and calorie restriction, as well as defined genetic mutations that increase lifespan, such as *indy* or *chico* (Rogina et al., 2000). Altogether, these tools make the fruit fly a suitable model to elucidate complex biological questions, such as the impact of the aging microenvironment on cancer. In recent years the significance of *Drosophila* for modeling cancer has also been established; four of the six hallmarks of cancer can be studied in the fly (the exceptions being telomerase activation and angiogenesis), and successful screens for the identification of genes important in metastasis have been implemented (Pagliarini and Xu, 2003; Woodhouse et al., 2003).

Drosophila is also an established model to study the involvement of heparan sulfate proteoglycans in signaling pathways (Lin, 2004; Lin et al., 1999) (Desbordes and Sanson, 2003) from a developmental context. We have already shown that Perlecan regulation of the growth factor Hedgehog, initially characterized in *Drosophila* neural stem cells (Park et al., 2003), is crucial for advanced prostate cancer proliferation (Datta et al., 2006a), thus validating the relevance of discoveries made in *Drosophila* in human prostate cancer. This makes *Drosophila* an ideal model in which to formulate testable hypotheses about the role of aging in the dynamics of tissue microenvironment composition and growth factor signaling, both factors that influence prostate cancer progression and outcome.

We have successfully shown that our studies on cell proliferation in the *Drosophila* CNS can translate to significant discoveries about signaling in prostate cancer. However, we still want to mimic as much as possible the context and microenvironment that occur in the prostate, a secretory gland in the male reproductive system. The use of an analog prostate organ in *Drosophila* is an attractive option to model age-dependent alterations

in microenvironment, specifically heparan sulfate proteoglycan function on signaling and proliferation.

Here, we present the Ejaculatory Bulb (EjB) as a potential prostate analog in *Drosophila*, which can be used as a model that incorporates aging, cancer, signaling, and microenvironment. I will discuss similarities in the function of the prostate and the EjB, as well as underlying correlations in the molecular blueprint of these two evolutionarily distant organs. I will also explore the dynamics of the microenvironment in the aging EjB, both in terms of HSPG and signaling pathway activity. Finally, we will discuss engineered overgrowth phenotypes and spontaneous overgrowth of the EjB, as well as possible strategies for modeling metastasis in this organ.

Materials and methods

Genetic strains and transgenes

Flies were grown in standard medium at 25°C unless otherwise stated. Markers and transgenes are described in Flybase.

Aging studies

All strains were allowed to develop at 25°C. Newly eclosed males were collected daily and placed in vials containing standard medium and supplemented with live yeast. Flies were then kept at 25°C or 18°C degrees and transferred every 3 days.

BrdU incorporation

Newly eclosed males raised at 25°C were starved for 2 hours by transferring them to empty vials. After 2 hours, flies were placed in tissue paper with 10% sucrose solution

containing 100mg/ml BrdU for 8h or 24h pulses. EjBs were then dissected and fixed as described (Park et al., 2003). BrdU incorporation was visualized with a mouse primary antibody (BD Biosciences) and a peroxidase conjugated secondary antibody (Jackson Immunoresearch). Diaminobenzidine (DAB) was used to developm the secondary antibody. EjBs were mounted on slides for visualizing with a Zeiss Axiophot compound microscope.

β-galactosidase enzymatic staining

Newly eclosed Ejaculatory bulbs were stained and fixed with 1X ET fix (1X Buffer B, 20% formaldehyde) for 10 minutes at RT. Tissues were washed with PBST and incubated with X-Gal stain for 2 hours at 37°C.

RNA isolation and RT-PCR

RNA was isolated using the RiboPure kit (Applied Biosystems), treated with DNAseI (Invitrogen), and reverse transcribed with oligo dT using the Superscript first strand reverse transcription kit (Invitrogen).

RNA amplification and real-time PCR

RNA was isolated using the RiboPure kit (Applied Biosystems) according to the manufacturer's instructions. For qRT-PCR, RNA was treated with DNAseI (Invitrogen), cleaned with the DNA-Free RNA kit (Zymo Research) and utilized in RNA amplification as previously published (Klebes et al., 2002). qPCR reactions were run on an iCycler (BioRad) using SYBR green Master mix (Applied bioystems). Primer sequences are available upon request.

Results

The Drosophila *ejaculatory bulb anatomy and function mirrors that of the human prostate*

In developing an analog organ model for the human prostate in *Drosophila*, we took into account the basic structure and function of the prostate. We then evaluated the male reproductive system of the fly for possible organs that have a similar structure and function. The human prostate is a male accessory reproductive gland that produces a complex proteolytic solution essential for sperm survival. It is located beneath the bladder, and completely surrounds the prostatic urethra (Figure 4.1A). The prostate is subdivided into 3 distinctive zones: peripheral, central, and transitional (Figure 4.1C). 70% of all prostatic carcinomas arise in the peripheral zone (Abel and Lanani, 2003). It is made up of epithelial glands and a fibromuscular stroma. The glandular epithelium (which gives rise to prostate adenocarcinoma) has 3 types of cells: basal, luminal secretory, and neuroendocrine. The stroma is composed of fibroblasts, smooth muscle cells, endothelial and dendritic cells, and infiltrating cells from the immune system (Feldman and Feldman, 2001).

The ejaculatory bulb (EjB) is a small organ in the *Drosophila* male reproductive system (Figure 4.1B), located in the male abdomen. It receives sperm from the testes, along with proteins from the accessory glands and aids in the pumping of seminal fluid through the ejaculatory duct, similar to the gross anatomy and function of the human prostate gland. The EjB also produces PEB-me, a protein that forms part of seminal fluid. The EjB is comprised of both muscle and secretory cells reflecting its dual function. While the prostate and EjB do not descend from a common ancestor organ, the correlation between their physiological functions and their specific anatomical connections in the male reproductive system suggest that there are might be significant similarity between these

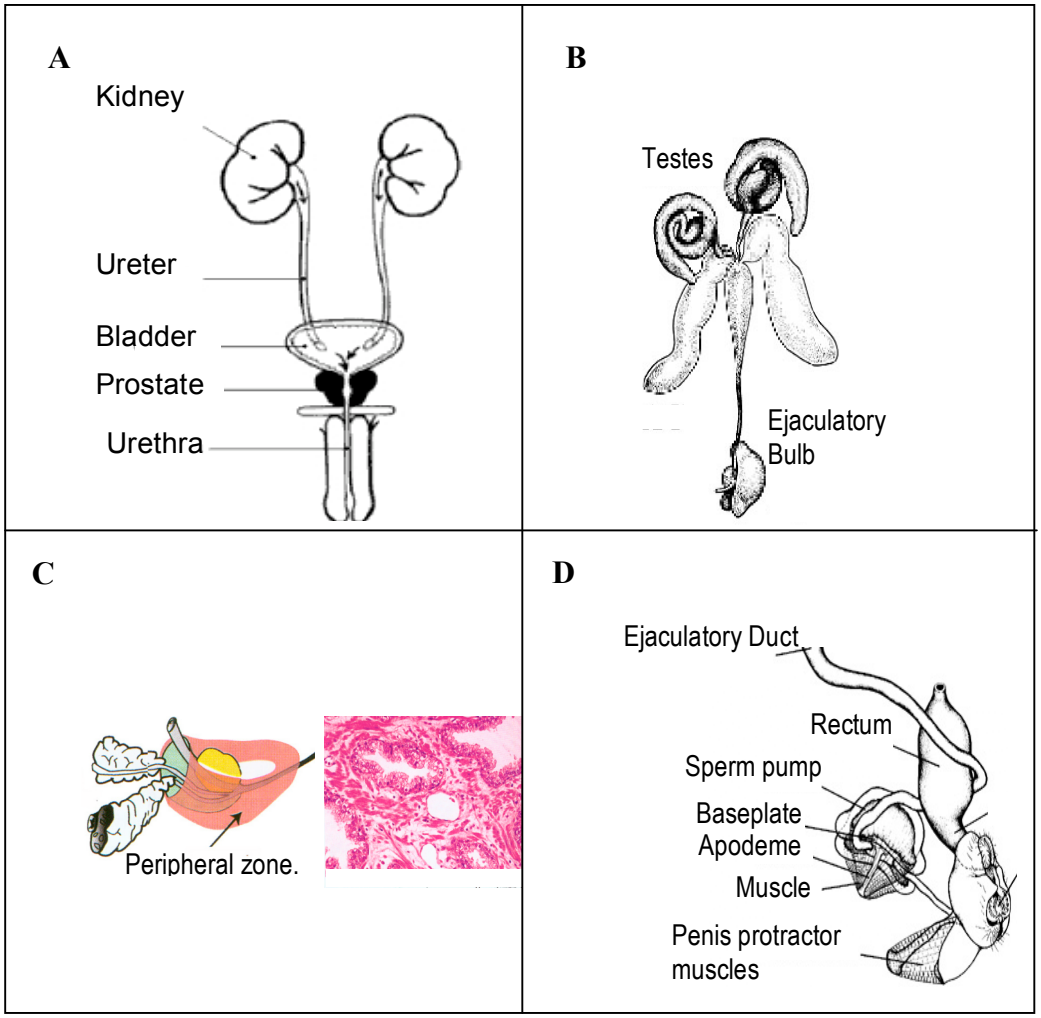


Fig.4.1. Anatomical and physiological connections of the prostate and ejaculatory bulb. (A,B). Location of (A) the prostate and (B) the Ejeaculatory bulb in the male reproductive system. (C). Differentiation of the three prostate zones and histology of the prostate.(D) detailed description of Ejaculatory bulb anatomy. Modified from and (Abel, 2001) the SEER training program (SEER Registry Database, 2008).

two systems. If this is so, then homologs of human or mammalian prostate-specific genes should be expressed in the *Drosophila* EjB

The EjB expresses prostate biomarkers

In order to assess the prostatic origin of cells, a number of genes are used in clinical and research studies as prostate-specific marker genes. These include transcription factors, such as *NKX.3* and *Pax2*, as well as the prostate-specific alkaline phosphatase gene *PAP* (Liu and True, 2002). In addition, several other genes have been proposed as biomarkers for prostate cancer. These genes are normally present in the prostate, but are abnormally upregulated during prostate cancer. These include Alpha-Methylacyl CoA Racemase (AMACR) and Sprouty-1, an inhibitor of FGF-2 signaling (Kuefer et al., 2002).We set out to identify putative *Drosophila* homologs using the Homologene database (Apatoff et al., 2006; Wheeler et al., 2001). Table 4.1 shows the prostate- specific and prostate cancer biomarkers, along with the *Drosophila* homologs. We evaluated expression of these genes in the EjB by RT-PCR. Figure 4.2 shows that the *Drosophila* EjB expresses the *Drosophila* homologs of human prostate-specific markers, as well as putative prostate cancer biomarkers. This finding strengthens the concept of a common molecular blueprint shared between these two organs, even in the absence of evolutionary homology. It also strengthens the possibility that signaling pathways relevant for prostate development and cancer are also active in the EjB.

Signaling pathways crucial for prostate development and cancer are present in the EjB

While androgen signaling in prostate development has been well characterized, there are a number of signaling pathways important for prostate development. The effects of Sonic Hedgehog (SHH), Fibroblast Growth Factor (FGF), Bone Morphogenetic Protein

Table 4.1.
Drosophila homologs of human prostate-specific and prostate cancer biomarker genes.

Human prostate	**Function**	***Drosophila* homolog**
NKX.3	Prostate Biomarker	*Bagpipe*
Pax 2	Prostate Biomarker	*Shaven*
Prostate specific alkaline phosphatase	Prostate Biomarker	*acph-1*
AMACR	Prostate cancer biomarker	*Amacr*
Sprouty-1	Prostate cancer biomarker	*Sprouty*

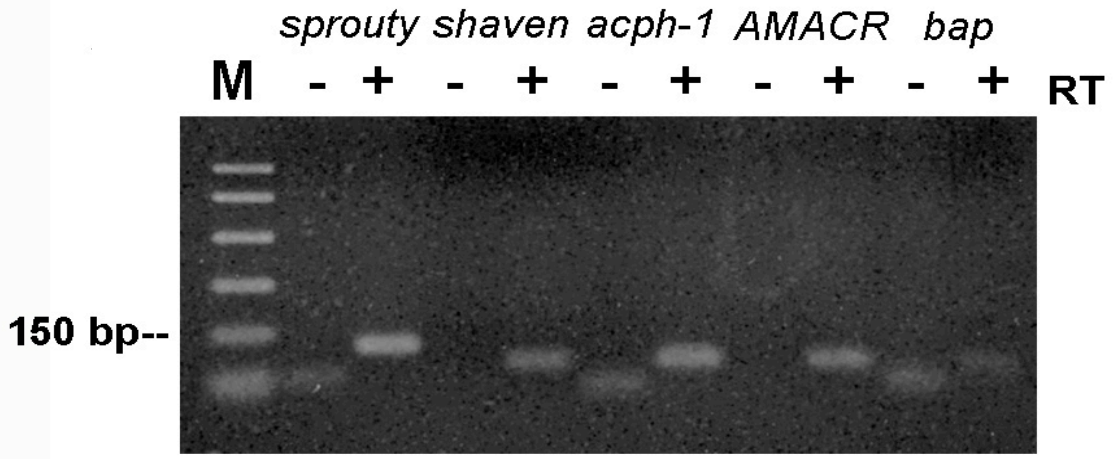


Fig. 4.2. The EjB expresses prostate-specific and prostate cancer biomarkers. (-) denotes no template controls and (+) denotes cDNA from Ejaculatory Bulbs.

(BMP), and Wingless-Int (Wnt) signaling pathways in normal or abnormal prostate development have been demonstrated **(**Yardy and Brewster 2005, Settle et al 2001, Kwabi-Addo et al 2004). Furthermore, the role of the SHH and FGF-2 signaling pathways in prostate development and cancer has been widely documented. These pathways are conserved in *Drosophila*, where Hh is homologous to SHH and Bnl is homologous to FGF-2 (Brook, 2000; Dawber et al., 2005; Lum et al., 2003; Sutherland et al., 1996). I first set out to evaluate the presence of active Hh, and Bnl signaling in the EjB by using transgenic lines expressing the *lacZ* gene in a pattern similar to *hh* or *bnl* expression. β-galactosidase activity staining revealed the presence of both the Hh and Bnl ligands in newly eclosed EjBs, as well as their respective response genes, *patched* and *pointed* (Figure 4.2). These results strongly suggest that there is active signaling by these growth factors in the EjB and raises the possibility that other components of the microenvironment known to regulate these two pathways are also present in the EjB.

Heparan sulfate proteoglycans (HSPGs) are an integral component of the extracellular matrix. It has been shown that the proteoglycans Syndecan and Perlecan play an important role in regulating both SHH and FGF-2 signaling both in embryonic development and in prostate development and cancer (Park et al., 2003; Spring et al., 1994; Tkachenko et al., 2005; Volk et al., 1999; Wu et al., 2001). In *Drosophila* there are 4 HSPGs; trol, syndecan, dally, and dally-like; which are homologs of human perlecan, syndecan, and glypicans respectively. We used RT-PCR to evaluate the expression of *Drosophila* HSPGs in the EjB. Our results (Fig 4.3) show that *trol*, the *Drosophila* perlecan homolog, and *sdc*, the *Drosophila* syndecan, are expressed in the EjB. Together with the presence of active Hh and Bnl signaling, the presence of HSPGs strongly suggests that major components of the prostate microenvironment relevant for prostate development and cancer are expressed in the EjB. It also raises the possibility

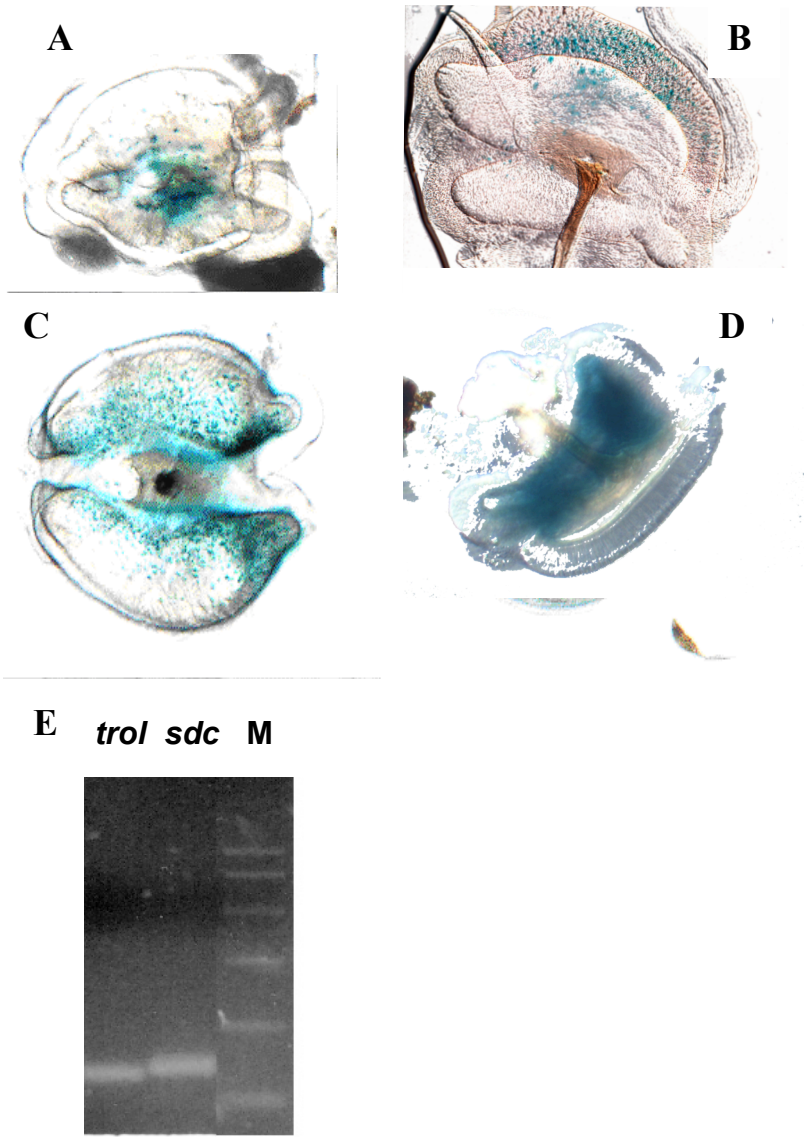


Fig. 4.3. The EjB presents active Hh and Bnl signaling, as well as *trol* and *sdc* expression. β-galactosidase staining of A. *hh-lacZ*, B. *bnl-LacZ*, C. *ptc-lacZ*, and D. *pnt-lacZ* Ejaculatory bulbs. E. EjB cDNA amplified with primers for *trol* (lane 1) and *sdc* (lane 2).

that HSPGs modulate growth factor signaling in the EjB like they do in the human prostate.

Age-dependent regulation of Hh signaling

SHH and FGF-2 signaling are normally downregulated in the adult prostate compared to developmental stages. We asked if a similar downregulation of Hh and Bnl signaling occurs in the adult *Drosophila* EjB as males age. Flies were aged at 25°C, and *Hh* and *bnl* expression and signaling activity was measured by qRT-PCR of ligands and response genes. Figure 4.4 shows that following eclosion, Hh signaling shows a steady decrease with time, as shown by the steady decrease in *ptc* expression, while Bnl signaling does not show significant changes with time, as shown by the levels of the response gene *pnt.* Moreover, for both Hh and Bnl, ligand expression does not mirror the level of response gene expression in aging flies, suggesting that regulation of growth factor signaling is not solely a factor of ligand concentration. From these results, we can conclude that Hh signaling in the EjB shows time-dependent decrease, whereas Bnl signaling does not.

Aging is measured in two different ways: chronological aging refers to the absolute time that has passed since the beginning of an event. Physiological aging refer to the changes in physiology that are normally associated with aging, and therefore is a more representative measure of the biological changes that we are investigating. To confirm that the changes in signaling are dependent on physiological aging, as opposed to chronological aging or time passed we carried out a separate aging scheme where flies were aged at 18°C. At this temperature lifespan is doubled without loss of normal metabolic rate or reproductive potential (Miquel et al., 1976). If changes in signaling are

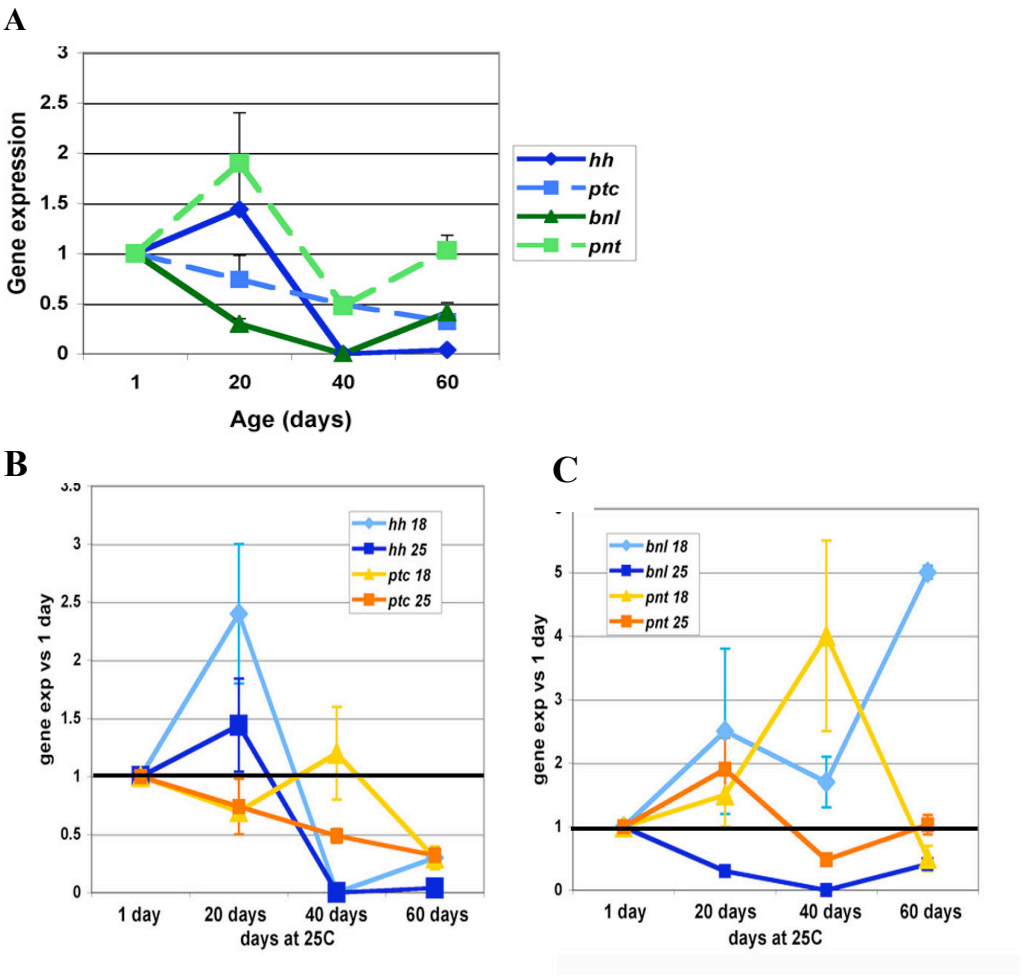


Fig. 4.4. Hedgehog signaling depends on physiological aging, while Branchless signaling does not. (A) Fold increase in expression of *hh, ptc, bnl* and *pnt* at 25°C. (B). Superimposed expression curves for hh and pat expression at 25°C and 18°C. (C) superimposed expression curves for bnl and pnt expression at 25°C and 18°C.

truly age dependent, changes in signaling will take twice as many days to become apparent in this second cohort. Figure 4.4 shows that at 18 degrees both *hh* and *ptc* expression decrease at approximately half the rate they do at 25°C. We see a strong standard deviation on our 40d data point, which is being reassayed. The overall pattern is consistent with Hh signaling being dependent on physiological aging. In contrast, the expression of *bnl* and *pnt* at 18°C does not appear to depend on physiological aging. We note that the decrease in Hh signaling with increasing age in the EjB does not parallel the decrease in expression of the Hh ligand. Other studies have also suggested that aged tissue is not as sensitive to growth factor signaling. We also know that Hh signaling is modulated by HSPGs. We therefore hypothesize that the downregulation in Hh signaling observed in the aged EjB is due to changes in the HSPG composition of the ECM. To evaluate changes in HSPG level in aging males, we used qRT-PCR. Figure.4.5 shows that following eclosion, the expression of *trol* (the mammalian Perlecan homolog) is strongly downregulated with time, while the expression of *sdc* (homolog of mammalian Syndecan) shows an initial strong decrease, followed by later upregulation. The expression of both *dally* and *dally-like* (homologs of mammalian glypicans) show a moderate decrease at 20 days following eclosion, a stronger decrease at 40 days, and upregulation towards the end of the adult life. These results show HSPG expression is dynamically regulated by time and that different proteoglycans show specific patterns of expression. It should be noted that the only proteoglycan that is strongly downregulated throughout the life span is *trol*, the homolog of human Perlecan.

We used flies aged at 18°C to determine if the changes in HSPG expression are dependent on physiological aging rather than time. Figure 4.5 shows that while *trol* and *dally* expression is age-dependent, syndecan and dally-like expression is not. It is important to note that both *trol* and *dally* have been implicated in Hh signaling in different systems. If misregulation of signaling results from changes in HSPG expression

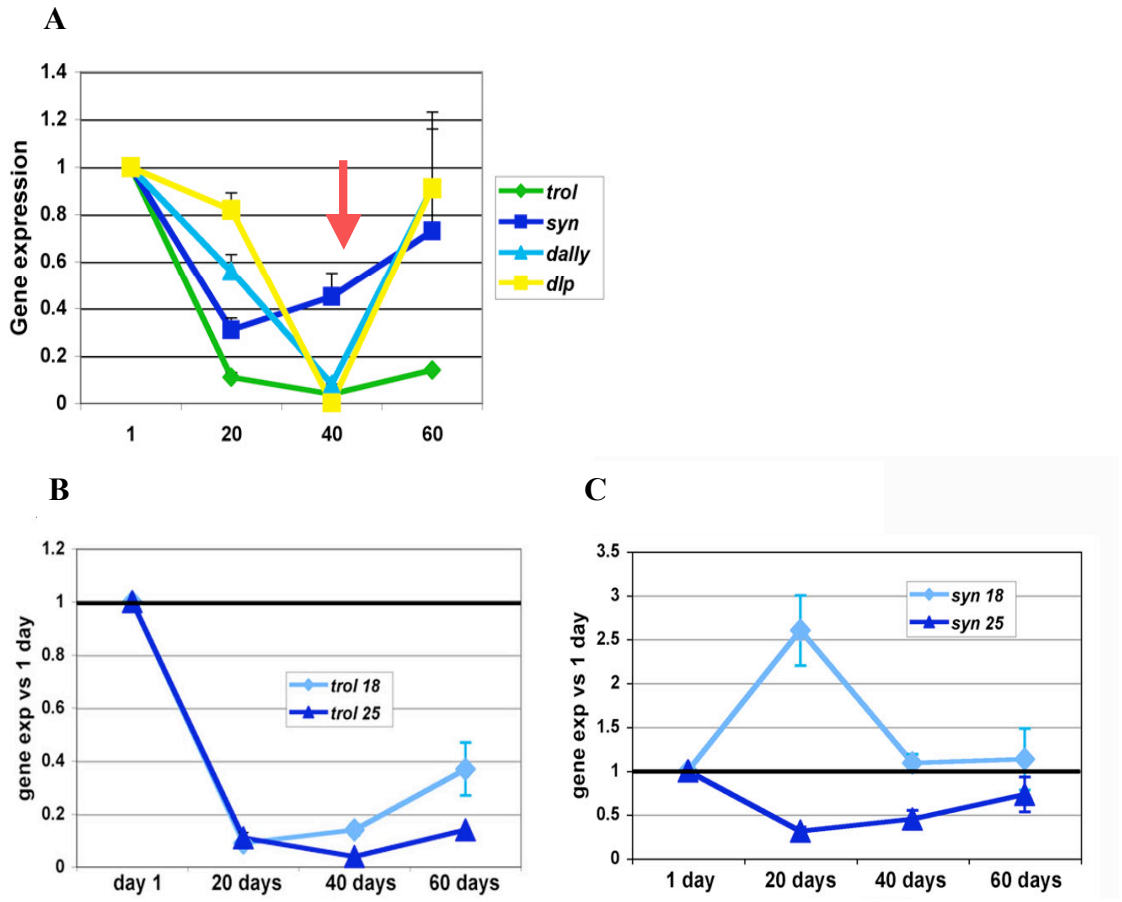


Fig. 4.5. Expression of *trol* and *dally* depends on physiological aging. (A) expression of HSPG at 25°C. (B) superimposed expression curves for *trol* expression at 25°C and 18°C. A similar trend was observed with *dally* expression. (C) superimposed expression curves for *syn* expression at 25°C and 18°C. A similar trend was observed for *dally-like* expression.

or composition, then age–related changes in HSPG should correlate with age- related changes in signaling activity. Our results have shown that the HSPGs Trol and Dally are regulated in an age-dependent manner, and that this correlates with Hh signaling in EjB, which is also age-dependent. Together with the fact that Perlecan regulates SHH signaling in prostate cancer, these results raise the possibility that Trol regulates Hh signaling in the aging EjB. Direct demonstration of this hypothesis requires manipulation of HSPG expression in the EjB and evaluation of any changes in signaling that may result**.**

Overgrowth of the EjB spontaneously with age and in response to expression of activated Ras

A key feature to the feasibility of the EjB as a model for prostate aging and cancer is the ability to recapitulate hyperproliferation, tumor formation and metastasis. As a first step, I decided to model hyperproliferation in the EjB by overexpressing a constitutively active form of the oncogene *Ras*. To this end, we used a heat-shock GAL4 driver crossed to a UAS-Ras^{v12} transgenic line. In order to minimize confounding developmental effects of Ras overexpression, the flies were raised at 18°C degrees, and transferred to 25°C upon eclosion. Figure 4.6 A shows the overgrowth phenotype obtained with 24h Ras^{v12} overexpression upon adult eclosion. The size of Ras^{v12} –overexpressing EjBs is 2-3 times larger than controls subjected to the same treatment. In order to test if the overgrowth phenotype is due to overproliferation of cells in the EjB, we assayed the levels of BrdU incorporation in the EjB. Surprisingly, we did found BrdU-incorporating cells in the adult EjB. This suggests that, although the vast majority of cells in *Drosophila* adult (with the exception of the germline) are postmitotic, there are a few cells in the EJB capable of proliferation. Whether these are the cells that produce the EjB overgrowth in response to Ras overexpression remains to be seen. Because our initial BrdU incorporation assay depended on food intake, the results can be obscured by differential feeding rates in different fly strains. To clarify the results obtained, we

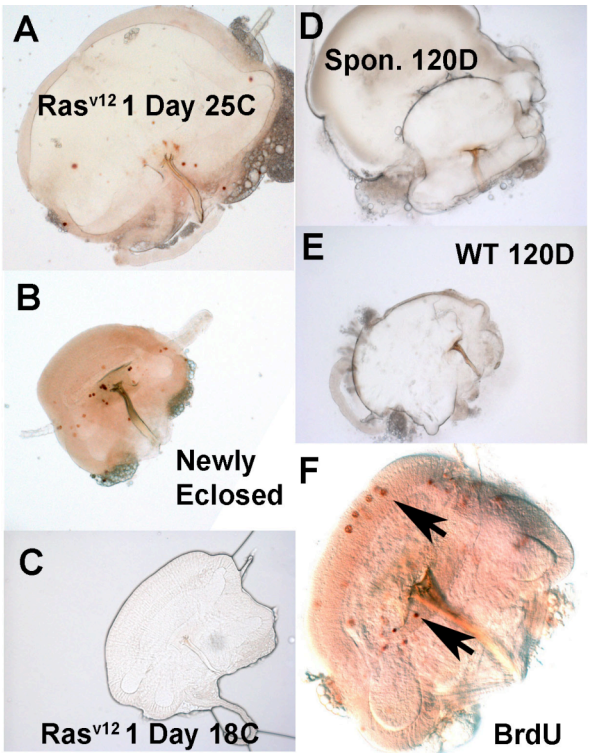


Figure 4.6. Overgrowth and proliferation in the EjB. A-C. Overgrowth on EjB with overexpression of Ras^{v12} (A) compared to controls (B,C). D,E. Spontaneous overgrowth observed in an 120d EjB aged at 18°C (D), compared to wild type (E).(F) BrdU incorporation in a newly eclosed EjB. Arrow indicates BrdU-incorporating cells.

have also developed a topical BrdU incorporation assay, as well as a cyclinE qPCR assay. These assays have yet to be tested.

In addition to the engineered transgenic overgrowth model, we have also seen spontaneous overgrowth of the EjB in one aged CS 120d adult raised at 18°C degrees. Figure 4.6C shows the spontaneous overgrowth phenotype. This sample raises the possibility of an age-dependent increase in spontaneous overgrowth, since we have never observed this phenotype in flies younger than 120d raised at 18°C degrees, or fliesyounger than 60 days raised at 25°C. I am currently pursuing the study of this spontaneous overgrowth phenotype to determine its frequency, statistical significance, and age dependency.

Progress toward understanding tumorigenesis in the EB

While the ability to engineer overgrowth phenotypes allows us to effectively model overproliferation in the EjB, our engineered Ras- overexpressing strain has a very short lifespan, probably owing to residual activity of the hs-GAL4 driver during all development stages. Also, because effects of hs-GAL4 are systemic, there may be confounding effects from Ras^{v12} overexpression in other organs that affect viability or EjB development and growth. This will impair the study of age-dependent changes in this strain. A possible solution involves driving overexpression specifically in the EjB of male flies. To this end, we have identified the protein PEB-me (Lung and Wolfner, 2001), which is specifically expressed in the EjB. We can then use the PEB-me promoter to construct a GAL4 driver that will only be active in the adult EjB alone. The GAL4 drive can then be coupled with the Tubulin-GAL80 construct, which will repress all GAL4 expression at the restrictive temperature. This will allow overexpression of Ras^{v12} exclusively at specific timepoints in the adult. We will also be able to use this system to

overexpress different HSPGs and directly evaluate the effects of altered microenvironment on signaling and proliferation in the aging EjB.

Discussion

Emerging research in the fields of aging and cancer has established a link between these two complex biological processes. The cellular microenvironment has been shown to play a fundamental role in cancer progression, as well as undergo dramatic changes with aging. Age is the most important risk factor for prostate cancer; however, opportunities to study this important risk factor *in vivo* are limited by the long lifespan and high cost of currently used models. These findings highlight the need for simple models in which to study the effects of the aging microenvironment on extracellular signaling, cell proliferation, and cancer progression. Here, we present the *Drosophila* Ejaculatory Bulb as a prostate analog organ that incorporates all these aspects in a simple model organism. We have shown that the EjB, a secretory organ of the *Drosophila* male reproductive system, exhibits functional similarity to the human prostate. An exciting discovery is that the EjB expresses prostate-specific biomarker genes and recapitulates components of the microenvironment found in the human prostate. This emphasizes the possibility that these two organs share a molecular blueprint, or deep homology, despite not being evolutionarily related.

We have shown that the EjB expresses signaling pathways present in the human prostate, as well as microenvironment HSPGs that regulate these pathways. We hypothesize that age-dependent alterations in HSPGs affect signaling strength and proliferation. My results show that the EjB undergoes age-dependent changes both in the strength of Hh signaling and in the expression of the HSPGs *trol* and *dally*. This suggests that both Hh signaling and *trol* and *dally* expression are regulated by physiological aging. This also raises the possibility that Trol regulates Hh signaling in the aging EjB, in a way similar to the mechanism of Hh signaling in the *Drosophila*

larval CNS and in prostate cancer. Further study is needed for direct demonstration of this hypothesis.

My studies of Bnl signaling suggest that there is no decrease of signaling strength with aging. However, our real-time PCR studies have been done on whole dissected EjBs. This means that there could be localized changes in signaling that we would not detect with this system. A way to refine this study would include immunohistochmistry for response proteins or in situ hybridization of response gene expression to reveal spatial and temporal patterns of change. Another possible explanation for the lack of downregulation in *pnt* expression is that this response gene may also receive input from other RTK signaling pathways such as EGFR. We can also use qPCR to determine the levels of other RTK ligands.

By overexpressing Ras^{v12} I have been able to engineer an overgrowth phenotype in the EjB. A potential spontaneous age-dependent overgrowth phenotype is also under study. I have also found BrdU-incorporating cells in the adult EjB, and have developed assays to effectively measure proliferation in the EjB. These findings show our ability to recapitulate overgrowth in the EjB, and give strength to the idea that the EjB can be used to model cancer progression. However, an effective model for cancer progression also needs to recapitulate tumor formation and metastasis. *Drosophila* metastasis screens have made use of Ras^{v12} overexpression to identify other genes important for tumorigenesis and metastasis. This approach has identified genes in the apico-basal polarity pathway, such as *scribble* or *discs large*, as a cause of metastasis in *Drosophila* larvae. Remarkably, the human homologs of these genes are fundamental in the epithelial to mesenchymal transition (EMT), a hallmark of cancer progression. EjB–specific over proliferation of Ras^{v12} can be a starting point to test if overexpression of different HSPG in the EjB results in overproliferation and tumor formation and thus recapitulate the formation of metastatic tumors. We can use Ras^{v12} overexpression, as well as mutations in *scribble*, in conjunction with HSPG overexpression to determine if

HSPG act as cooperating oncogenes. We can also use this approach to identify new potential cooperating oncogenes in the EjB.

My studies on the *Drosophila* Ejaculatory Bulb have uncovered deep homology with the human prostate, making it a feasible prostate analog organ. I have also tested the hypothesis that age-dependent changes in the microenvironment affect signaling, and found that Hedgehog signaling is regulated by age, My data suggests a correlation bwtween age-dependent changes in *trol* and *dally* expression and the age regulation of Hh signaling This raises the possibility that Hedgehog signaling in the aging Ejaculatory bulb is regulated by the heparan sulfate proteoglycans Trol and Dally, although direct demonstration of this regulation is still needed. My progress towards modeling tumorigenesis and metastasis in this model includes the engineering of an overgrowth phenotype by overexpressing the oncogene Ras, and development of methods for measuring rates of proliferation in the Ejaculatory Bulb. I have also observed a possible spontaneous overgrowth phenotype raising the posiibility of studying age-dependency of overgrowth. Altogether, these results show that the drosophila Ejaculatory bulb emerges as a promising model to study the effects of aging on microenvironment, signaling, overproliferation, and cancer progression.

CHAPTER V
CONCLUSIONS AND FUTURE DIRECTIONS

The general theme of my research is the importance of model organisms in understanding and elucidating complex biological processes of clinical relevance. I have focused my research on the mechanisms underlying the proliferation and metastasis of advanced prostate cancer. Based on a model of proliferation previously characterized in the fruit fly *Drosophila*, I hoped to gain insight about the role of the microenvironment in prostate cancer. Specifically I have focused on the role of the heparan sulfate proteoglycan PERLECAN in regulating the growth factor Sonic Hedgehog (SHH) to ultimately control proliferation in advanced prostate cancer. This knowledge would not only signify a greater understanding in a previously obscure mechanism for prostate cancer proliferation; it would also highlight the relevance of emerging areas of research with either proven importance or high correlation with cancer progression. Research in these areas is bringing about the discovery of new biomarkers and drug targets, as well as refining our current paradigm on prostate cancer. Just as importantly, my studies validate the use of the fruit fly *Drosophila* to formulate and test initial hypotheses about mechanisms of clinical relevance that can then be validated in human disease.

The management of disease consists of several important steps, which include prevention, accurate monitoring and diagnosis of the disease as well as accurate prognosis of the outcome/development of the disease. An integrated analysis of the disease will produce the most useful approach in choosing/ developing treatment strategies. An initial step of crucial importance is the development of biomarkers for large-scale screening. Implementation of routine testing for currently available biomarkers, such as Prostate-specific Antigen (PSA) (Brawer et al., 1992) has greatly increased the diagnosis of prostate cancer. However, a significant fraction of PSA

screening-detected prostate cancer would not develop into clinical symptomatic prostate cancer, and therefore it would not have an impact on mortality (Gelmann, 2008). This fact highlights the need to find strategies that will target aggressive forms of prostate cancer, as opposed to slow-progressing prostate cancer. Emerging biomarkers include SPROUTY-1, which shows downregulation in 40% of prostate tumors (Kwabi-Addo et al., 2004), and Alpha methylacyl CoA racemace (AMACR) which has been successfully utilized as a biomarker for colon cancer and correlates with clinically localized prostate cancer (Kuefer et al., 2002). SROUTY-1 is an inhibitor of the FGF pathway, and AMACR expression has been shown to correlate with androgen signaling. This highlights the importance of studying the clinical implications of extracellular signaling pathway function and deregulation. The need remains for a biomarker that correlates positively with aggressive behavior, and can be used to screen specifically for aggressive prostate cancers.

Current treatment strategies for prostate cancer include surgery and radiation. However, in the event of invasive prostate cancer, or presence of metastasis, these treatments are not effective. Androgen ablation therapy is then used as a treatment; reducing the supply of androgens to the tumor causes it to slow growth, but it also has significant side effects. The value of this treatment is only partial, since prostate cancer cells inevitably become refractory to this treatment, and patients die from androgen-independent prostate cancer metastases. This raises the possibility that other types of growth factor signaling can maintain and support cancer growth and proliferation in the absence of androgen signaling. In this case, targeting growth factor signaling alone or in combination with androgen ablation therapy might reduce the chances of androgen-independent tumor regrowth, and reduce mortality rates.

The process of metastasis includes the regrowth of an entire tumor in a location removed from the original tumor site. This implies that a single cell or a small group of cells have the capacity to regenerate the original tumor phenotype. In this context, a tumor-

regenerating cell can be considered as a stem cell, and development of treatment strategies will benefit from knowledge of stem cell biology. Stem cell proliferation is tightly controlled and regulated by a combination of environmental factors, the stem cell niche. These niches are rich in growth factors and other signaling molecules, and in their absence stem cells might differentiate or arrest growth. This raises the possibility that targeting regulators of stem cell proliferation will be a useful strategy against metastatic tumors, since it will prevent or inhibit tumor regrowth.

The cancer stem cell theory underlines the importance of considering cancer as an integrated system, one that considers not only deregulation within the cell but also alterations in the extracellular microenvironment. Stem cell proliferation is a complex biological process, where the stem cell has specific characteristics that may depend on the extracellular niche. Another example of integrative biology is looking at cancer from a developmental point of view. In development, different cells interact to produce specific cell fates, and many extracellular signals have to be precisely coordinated to result in organized proliferation, cell migration, and differentiation. All of these approaches emphasize the important and contextual role of the extracellular microenvironment in cancer.

Our main goal was to focus our studies on processes that not only provide mechanistic understanding of prostate cancer progression, but that can be translated into discoveries with clinical consequence. Therefore I focused on the study of the microenvironment's role in advanced prostate cancer. A better understanding of the microenvironment contribution in the progression to advanced disease would highlight the importance of the extracellular context in the proliferation of cancer cells. It will also provide us with promising new drug targets or biomarkers that specifically target the more aggressive forms of prostate cancer.

Prostate cancer is the leading cause of death from cancer in American men. Last year there were approximately twenty-seven thousand men killed by this disease. Due to its extensive incidence and mortality, prostate cancer has been studied extensively. Identified risk factors for prostate cancer include age, race, and family history (Zheng et al., 2008). The contribution of environmental factors, while acknowledged, is less clear (Kristal et al., 2006). Human genetics studies have linked many loci to increased risk of prostate cancer. Among these is the CABP locus, located in 1p36, which correlates with increased risk of both prostate and brain cancer (Gibbs et al, 1999). One of the genes that map to this locus is *PERLECAN*, which encodes a heparan sulfate proteoglycan on the extracellular matrix. *PERLECAN* is expressed abnormally in other types of cancer, such as melanoma (Cohen et al., 1994; Sharma et al., 1998) and is upregulated in the prostate cancer cell line PC3 (Iozzo et al., 1994). In the model system *Drosophila*, PERLECAN has been shown to be required for growth factor-dependent proliferation of quiescent neural stem cells. These findings raise the possibility that heparan sulfate proteoglycans and developmental growth factor signaling regulate proliferation in advanced prostate cancer.

Several risk factors for prostate cancer have been studied successfully in different model systems, such as prostate cell lines, human tissue, and mouse models of tumorigenesis. Lower model organisms such as the fruit fly *Drosophila* offer a useful balance between that simplicity that permits straightforward analysis and the complexity that allows investigation of multifaceted phenomena. The genetic simplicity and sophisticated genetic and molecular tools available in *Drosophila* permit a clear interpretation of the results, while the fact that analysis is carried out in a living organism allows complex biological processes to be elucidated. The evolutionary distance between flies and humans is a factor that may be overcome because of deep homology, and its short lifespan and easy cost- effective handling is a distinct advantage over mouse models of carcinogenesis. Therefore I have chosen to elucidate basic mechanisms of carcinogenesis in flies and validate those findings in human samples.

I hoped to use the knowledge gained from previous studies in *Drosophila* to further understand the role of the microenvironment in advanced prostate cancer. This would elucidate potential mechanisms of disease progression, as well as uncover potential drug targets. If knowledge from *Drosophila* can be validated in the context of human prostate cancer, then *Drosophila* can also be used as a system in which to study different risks factor for prostate cancer, and ultimately elucidate their predictive, causative, and diagnostic value.

For example, previous studies in the *Drosophila* central nervous system uncovered a role for the HSPG Trol, homolog to human PERLECAN in neural stem cell proliferation (Datta, 1995). Further studies showed that Trol modulates neural stem cell proliferation by modulating signaling by the growth factors Hh and Bnl (Park et al., 2003). The human homologs of Hh and Bnl, SHH and FGF-2 respectively, have been widely implicated in cancer in a number of tissues. Furthermore, both SHH and FGF-2 play a role in human prostate development (Berman et al., 2004). I hypothesized that PERLECAN functions in a similar manner in the prostate, and that it regulates growth factor signaling to ultimately control prostate cancer proliferation.

Our results show that human PERLECAN regulates SHH-dependent proliferation in advanced prostate cancer. We showed that there is a positive correlation between prostate tumors and upregulation of both PERLECAN and SHH. Also, we have shown that these two components colocalize in prostate tumors. We then manipulated the system using either primary tumor cultures, or prostate cancer cell lines. Our results show that PERLECAN–dependent SHH signaling has a significant effect on proliferation.

Perspectives

Based on the observed genetic link between increased risk for both prostate and brain cancer at the CABP locus, and the fact that the human *PERLECAN* gene maps to the same location, we hypothesized that *PERLECAN* might be the CAPB locus gene, and play a significant role in prostate cancer. Based on our previous knowledge about PERLECAN mode of action in *Drosophila*, we proposed that the mechanism for PERLECAN action in advanced prostate cancer involved regulation of the SHH signaling pathway in prostate tumors. As part of a collaborative study, I have shown that Sonic Hedgehog (SHH) signaling is necessary for tumor proliferation in advanced prostate cancer, and that PERLECAN regulates SHH- dependent proliferation in advanced prostate cancer. I have uncovered two mechanisms for PERLECAN regulation of SHH signaling in prostate cancer cells: The first one depends on upregulation of *PERLECAN* expression, and the other involves alterations in PERLECAN composition/structure that allow for better binding of SHH. My studies have uncovered a significant role for deregulation of developmental signaling as well as an important contribution of the microenvironment in advanced prostate cancer.

It has been proposed that the normal direction of SHH signaling, from epithelial cells to the mesenchyme is altered in prostate cancer. In this case, epithelial cells would themselves be able to respond to SHH, and activate uncontrolled proliferation. There are conflicting results concerning this hypothesis. A possible explanation for these conflicting results involves taking a closer look at the microenvironment, in which case an examination of PERLECAN levels in the cells/media might shed some light on the mechanisms involved.

My results have emphasized the important role of the microenvironment in prostate cancer in terms of SHH and PERLECAN, uncovering two potential drug targets and

biomarkers. My work on an androgen dependence to androgen- independence cell line model also raises the possibility that cancer cells may be switching from androgen signaling to signaling by SHH and possibly other growth factors, as a means to sustain growth and proliferation (Figure 5.1). In this case, PERLECAN plays a role as a regulator of extracellular signals present in the microenvironment (Figure 5.2). In this context, the ability of cells to respond to the low concentrations of growth factors present in the epithelium will be greatly influenced by the levels/composition of PERLECAN present. In this context, inhibition of PERLECAN function could be an effective target for treatment strategies of metastatic prostate cancer.

As I pointed out earlier, the inherent heterogeneity of prostate cancer creates a necessity to tailor screening and treatment strategies for subsets of tumors. The greatest priority would be the highly invasive and metastatic tumors. The most commonly used biomarker used for early detection is PSA, yet it has shown no benefits from early detection in patients with a Gleason Grade of 7 or higher (D'Amico et al., 2001). Therefore, establishment of molecular markers that correlate with high Gleason score would be a useful. PERLECAN correlates positively with a Gleason score of 6 or higher (Datta et al., 2006a), suggesting a potential to be developed as a marker for aggressive prostate tumors.

SHH signaling has been shown to play a role in other types of cancer, including small lung (Watkins et al., 2003), brain tumors, pancreatic cancer (Thayer et al., 2003) and gastric adenocarcinoma (Ma et al., 2005). Notably SHH signaling plays an important role in the normal development these organs. In some of these cases, such as glioma, the transcription factor GLI1 is altered which causes active SHH signaling even in the absence of the SHH ligand. But in many other cases, the activity of the SHH pathway depends on the presence of SHH ligand (Ma et al., 2006). This raises the possibility that PERLECAN might also be present in these tumors, and that it is regulating the aggressiveness of the phenotype. A closer study of the microenvironment in these types

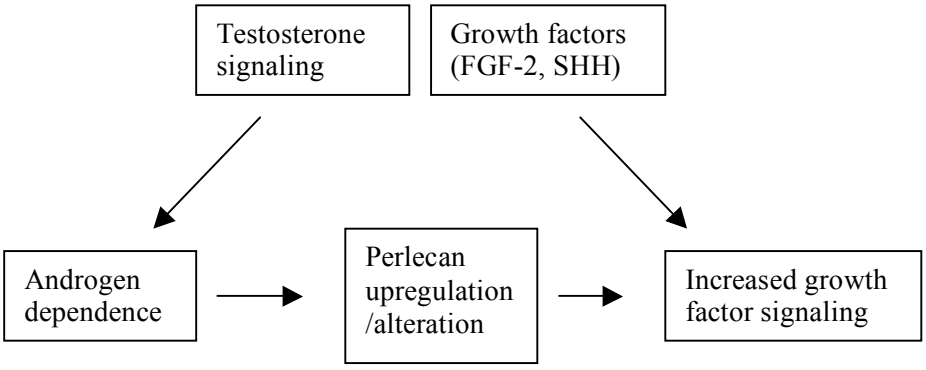


Fig. 5.1. From androgen signaling to growth factor signaling. Schematic diagram representing the role of perlecan in swithching to growth factor signaling.

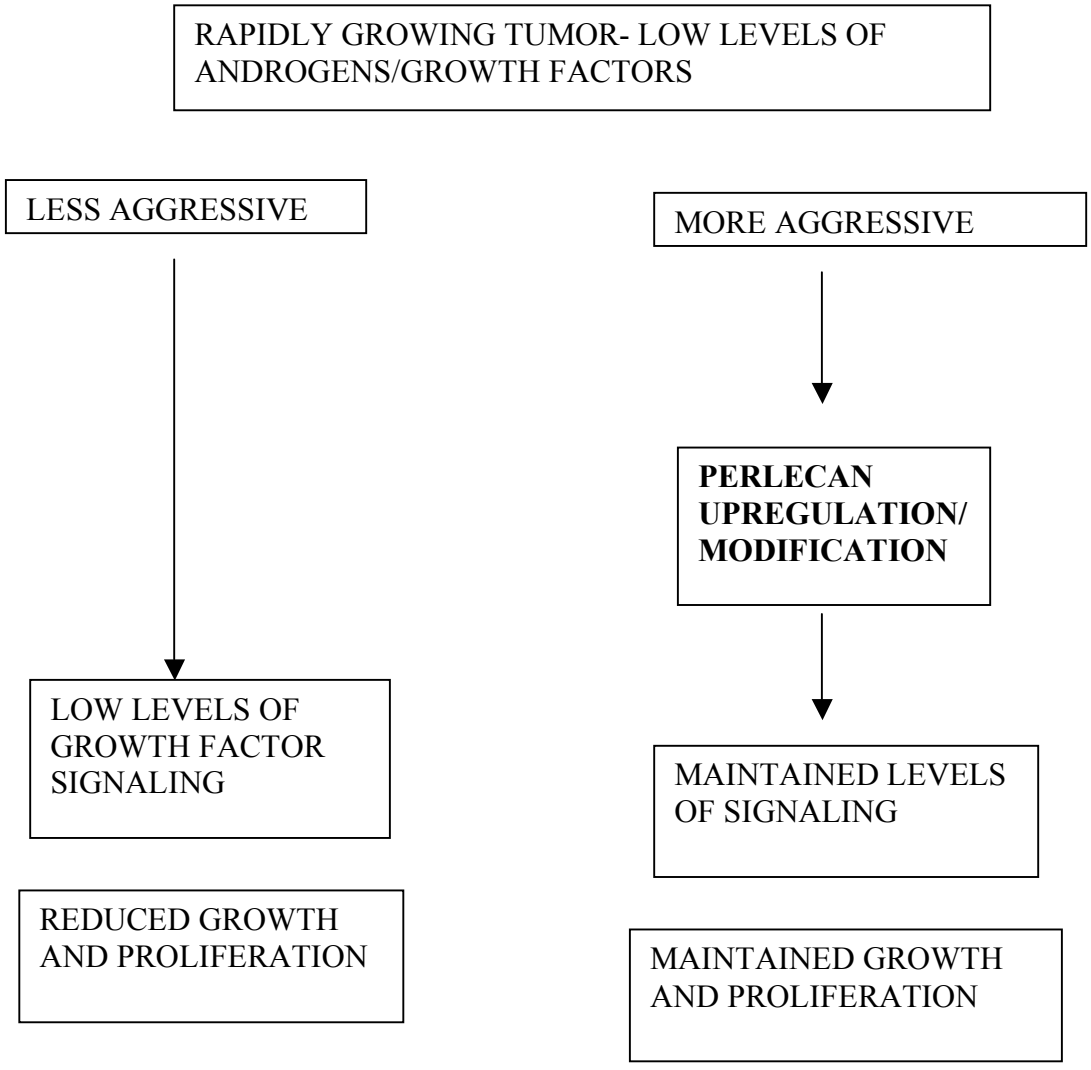


Fig. 5.2 model of PERLECAN regulation in the microenvironment for maintained signaling and proliferation.

of cancer will give us insight into whether PERLECAN regulation of SHH signaling is a common feature of these types of aggressive cancer. This raises the possibility that deregulation of developmental signaling in cancer, a common feature of all these types of tumors, is being mediated by the cellular microenvironment, notably by heparan sulfate proteoglycans such as PERLECAN.

The fact that PERLECAN regulation of SHH–dependent proliferation is conserved from *Drosophila* to humans highlights the role of conserved signaling mechanisms in processes of clinical relevance. This type of result is a strong validation of the use of a lower organism such as *Drosophila* as a starting point for formulating and testing hypotheses about other risk factors that might be relevant to human prostate cancer (Figure 5.3).

Among the risk factors for prostate cancer is a diet rich in cholesterol. Prostate cancer incidence has been rising steadily since the 1950's according to the American Cancer Society, and this increment is partly due to environmental factors. It has been shown that populations with traditional low incidences of prostate cancer (such as the Japanese and Chinese) experience a sharp increase in incidence upon migration to sites where prostate cancer incidence is high (Abel and Lanani, 2003), indicating that external factors play a role. It is thought that one of these external influences is diet; the western diet is rich in saturated fat and cholesterol. Recent reports have shown a positive correlation between high cholesterol levels (hypercholesterolemia) and prostate cancer (Bravi et al., 2006). Cholesterol has also has been shown to augment cell survival in prostate cancer models (Zhuang et al, 2005). Interestingly, cholesterol is an important modifier of a variety of signaling molecules, such as SHH and Wnt, which are connected to prostate cancer. This raises the possibility that nutritionally supplied cholesterol levels may have an effect on

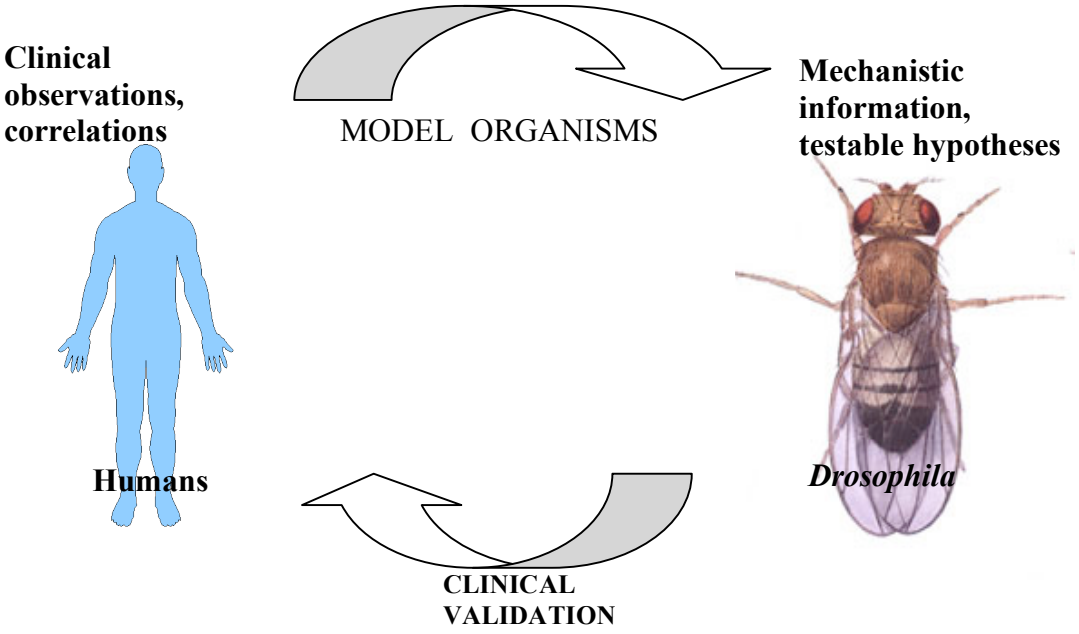


Fig. 5.3 From humans to *Drosophila*. Summary of how model organisms can be used to gain mechanistic insights, and validate them in human systems in order to dicover new drug targets/biomarkers.

prostate cancer cell survival due to an increase in the formation of cholesterol-modified SHH signaling. Cholesterol modification is an important step in the processing of mature SHH ligand (Zeng et al., 2001). All Hh proteins are produced as preproteins, which undergo autocatalytic cleavage. At this time, a cholesterol adduct is added to the N-terminus, which forms the active signaling portion. The fully processed form of SHH also undergoes palmitoylation after cholesterol modification. The importance of cholesterol and palmitoyl modifications is conserved from *Drosophila* to humans, and initial inferences about the role of cholesterol modifications have been characterized in *Drosophila*. Initially, it was thought that the unmodified N-terminal portion of Hh, termed HhN, retained full signaling activity (Porter et al., 1995). Further studies have identified a role for cholesterol modification in modulating SHH signaling strength (Porter et al., 1996b) (Karpen et al., 2001) and range (Zeng et al., 2001) (Wendler et al., 2006) (Dawber et al., 2005). The precise mechanism for lipid-modified Hedgehog remains a subject of controversy. There is also strong evidence for the important role of heparan sulfate proteoglycans (HSPG) in the adequate relay of the Hh signal (The et al., 1999).

In the *Drosophila* CNS, Hh acts as an activator of neural stem cell proliferation. To elucidate the importance of cholesterol modification for Hh signaling in this system, we overexpressed HhN in the first instar larval brain. To our surprise, HhN acted as an inhibitor of neuroblast proliferation in a dose-dependent manner. We hypothesized that HhN might be acting as a competitive inhibitor of cholesterol-modified Hh. To test this hypothesis, we first set out to evaluate the levels of Hh signaling in the event of HhN overexpression. My results show a negative feedback loop in Hh signaling, where *hh* is expressed in accordance to the levels of Hh signaling present. In this context, HhN acts as a Hh analog, and causes a decrease in the levels of endogenous Hh signal. This preliminary results need to be further evaluated in terms of the mechanism for the proposed Hh negative feedback loop as discussed in Chapter II.

We have shown that unmodified and cholesterol- modified forms of Hh have different effects in *Drosophila* neural stem cell proliferation. My results suggest a possible mechanism for this difference, and have uncovered a negative feedback loop that appears to regulate levels of Hh signaling in the *Drosophila* brain. These results raise the possibility that a similar mechanism might be in place in prostate cancer cells. While this avenue of research will provide mechanistic insight in terms of disease progression, in terms of clinical significance the effect might be of lesser impact. One reason for this is that high levels of cholesterol are a risk factor for a variety of other diseases, notably cardiovascular disease, the number one cause of mortality in men and women in the United States (Mehra, 2007). Given the clinical importance of cardiovascular disease, a significant number of the population already undergoes preventive screening for high cholesterol levels. Management of cholesterol levels is also extensive with dietary restrictions and treatment with statins, which lower cholesterol levels. Since prostate cancer is a disease of older men, and risk for cardiovascular disease presents at a younger age, clinical factors associated with risk for cardiovascular disease cause potential patients to be on treatment for high cholesterolemia by the time it might become relevant for prostate cancer.

Age is the principal risk factor for prostate cancer (Bostwick et al., 2004). A number of factors render the aging prostate more susceptible for cancer; these include an increase in genomic stability, oxidative and inflammatory stress, and alterations in the prostate microenvironment (Bavik et al., 2006). It is important to gain understanding of the mechanisms that cause the aging prostate microenvironment to have increased susceptibility to prostate cancer. This may uncover potential signaling mechanisms or microenvironment components that play a significant role in this process. These could have potential as clinical biomarkers, or drug targets. Recent studies in model organisms suggest that restoring normal aging-related signaling in adult animals has a significant

effect on lifespan (Dillin et al., 2002). This raises the possibility that the aging prostate microenvironment can be manipulated to restore maintenance.

Recent research in both the fields of aging and cancer has uncovered a possible link between these two processes. Although research in this new field has emerged only recently, there have already been reports of the contribution of the microenvironment to both aging and cancer progression (Krtolica et al., 2001). Elucidating the mechanisms underlying aging, and the impact of this factor on the processes such as cancer presents special requirements in a model. Cell lines have been successfully used to study the effects of senescent components in proliferation and growth. Yet this *in vitro* system is not well suited for recapitulating the complex process of aging, and its impact on tumorigenesis/ metastasis.

Mammalian models for prostate cancer include rat models that have been successfully used to study spontaneous tumorigenesis, as well as transgenic mouse models that have specific mutations in oncogenes or tumor suppressors. This allows for the elucidation of the function of these specific genes in vivo in the process of tumorigenesis. A disadvantage of mammalian models when studying the contribution of aging in tumorigenesis is the long lifespan of mice and rats, in the order of years, This makes the study of mutations affecting the lifespan and aging in mammalian systems, and their role on cancer development and progression costly and time- consuming. Lower organisms, such as *C.elegans* and *Drosophila* have been extensively studied in terms of development, and are established models for aging. *Drosophila* has also been used to model carcinogenesis and metastasis. This makes *Drosophila* an ideal system for studying the aging microenvironment and its impact in cancer progression. In the specific case of prostate cancer, it would be useful to have an organ analogous to the human prostate to model the impact of different factors in an organ-specific manner.

I have proposed a system to model prostate cancer in *Drosophila*. I have shown that the *Drosophila* Ejaculatory Bulb (EjB), an organ of the male *Drosophila* reproductive system, shows deep homology with the human prostate, and recapitulates microenvironment components and growth factor signaling that have been shown to be important in prostate cancer. I have also shown that expression of *trol* (the *Drosophila* Perlecan homolog) and Hedgehog signaling are regulated by physiological aging in the EjB. My progress towards modeling tumorigenesis in the EjB includes the engineering of an overgrowth model using overexpression of the oncogene Ras, as well as a possible spontaneous overgrowth phenotype in aged flies.

An important validation of this model is the ability to recapitulate tumorigenesis, invasion, and metastasis. In other *Drosophila* models of metastasis, this has been achieved by using a double-mutation screening strategy (Pagliarini and Xu, 2003). The system is sensitized by overexpression of an oncogene such as Ras, and then screened for secondary mutations that will result in a metastatic phenotype. This approach has uncovered genes in the apico-basal polarity pathway that lead to metastatic behavior. With this previous knowledge, a double mutant strategy can be used to test if tumorigenesis and metastasis can be modeled in an EjB-specific manner. To this end, an EjB-specific promoter has been engineered, which can be utilized to drive expression of different genes specifically in the EjB. Future directions of this project include testing the double mutant strategy and its effectness at modeling tumorigenesis and metastasis.

In summary, my studies have offered insight into application of the emerging paradigms in cancer to obtain clinically relevant results and integrating them into a new model for prostate cancer. The contribution of heparan sulfate proteoglycans in regulation of growth factor signaling might be a common mechanism in cancer progression, and it highlights the importance of the microenvironment as a drug target. My studies also validate the use of *Drosophila* as a system in which to formulate initial hypotheses about mechanisms that affect processes of clinical relevance.

REFERENCES

Abate-Shen, C., Shen, M. M., 2000. Molecular genetics of prostate cancer. Genes Dev. 14**,** 2410-2434.

Abel, P. D., Lanani, El-Nasir, 2001. Prostate cancer: Clinical and scientific aspects: bridging the gap. Imperial College Press, London.

Agren, M., Kogerman, P., Kleman, M. I., Wessling, M., Toftgard, R., 2004. Expression of the PTCH1 tumor suppressor gene is regulated by alternative promoters and a single functional Gli-binding site. Gene. 330**,** 101-114.

Akam, M., 1987. The molecular basis for metameric pattern in the *Drosophila* embryo. Development. 101**,** 1-22.

Alexander, R. B., Maguire, M. G., Epstein, J. I., Walsh, P. C., 1989. Pathological stage is higher in older men with clinical stage B1 adenocarcinoma of the prostate. J Urol. 141**,** 880-882.

Apatoff, A., Kim, E., Kliger, Y., 2006. Towards alignment independent quantitative assessment of homology detection. PLoS ONE. 1**,** 113.

Artavanis-Tsakonas, S., Rand, M. D., Lake, R. J., 1999. Notch signaling: cell fate control and signal integration in development. Science. 284**,** 770-776.

Artavanis-Tsakonas, S., Simpson, P., 1991. Choosing a cell fate: a view from the *Notch* locus. Trends in Genetics. 7**,** 403-408.

Bai, C. B., Joyner, A. L., 2001. Gli1 can rescue the *in vivo* function of Gli2. Development. 128**,** 5161-5172.

Bale, A. E., Yu, K. P., 2001. The hedgehog pathway and basal cell carcinomas. Hum Mol Genet. 10**,** 757-762.

Barnett, D. H., Huang, H. Y., Wu, X. R., Laciak, R., Shapiro, E., Bushman, W., 2002. The human prostate expresses sonic hedgehog during fetal development. J Urol. 168**,** 2206-2210.

Barrett, A., and Datta, S. 2007. Instability of *GAL4* drivers in the *Drosophila* larval brain. Unpublished report, Datta lab, Texas A&M University, College Station.

Bavik, C., Coleman, I., Dean, J. P., Knudsen, B., Plymate, S., Nelson, P. S., 2006. The gene expression program of prostate fibroblast senescence modulates neoplastic epithelial cell proliferation through paracrine mechanisms. Cancer Res. 66**,** 794-802.

Bellaiche, Y., The, I., Perrimon, N., 1998. Tout-velu is a *Drosophila* homologue of the putative tumour suppressor EXT-1 and is needed for Hh diffusion. Nature. 394**,** 85-88.

Berman, D. M., Desai, N., Wang, X., Karhadkar, S. S., Reynon, M., Abate-Shen, C., Beachy, P. A., Shen, M. M., 2004. Roles for Hedgehog signaling in androgen production and prostate ductal morphogenesis. Dev Biol. 267**,** 387-398.

Berman, D. M., Karhadkar, S. S., Hallahan, A. R., Pritchard, J. I., Eberhart, C. G., Watkins, D. N., Chen, J. K., Cooper, M. K., Taipale, J., Olson, J. M., Beachy, P. A., 2002. Medulloblastoma growth inhibition by hedgehog pathway blockade. Science. 297**,** 1559-1561.

Berman, D. M., Karhadkar, S. S., Maitra, A., Montes De Oca, R., Gerstenblith, M. R., Briggs, K., Parker, A. R., Shimada, Y., Eshleman, J. R., Watkins, D. N., Beachy, P. A., 2003. Widespread requirement for Hedgehog ligand stimulation in growth of digestive tract tumours. Nature. 425**,** 846-851.

Berman, J. R., Kenyon, C., 2006. Germ-cell loss extends C. elegans life span through regulation of DAF-16 by kri-1 and lipophilic-hormone signaling. Cell. 124**,** 1055-1068.

Bissell, M. J., Radisky, D., 2001. Putting tumours in context. Nat Rev Cancer. 1**,** 46-54.

Blaess, S., Corrales, J. D., Joyner, A. L., 2006. Sonic hedgehog regulates Gli activator and repressor functions with spatial and temporal precision in the mid/hindbrain region. Development. 133**,** 1799-1809.

Blasco, M. A., 2007. Telomere length, stem cells and aging. Nat Chem Biol. 3**,** 640-649.

Bonkhoff, H., 1996. Role of the basal cells in premalignant changes of the human prostate: a stem cell concept for the development of prostate cancer. Eur Urol. 30**,** 201-205.

Bonnet, D., Dick, J. E., 1997. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat Med. 3**,** 730-737.

Bornemann, D. J., Duncan, J. E., Staatz, W., Selleck, S., Warrior, R., 2004. Abrogation of heparan sulfate synthesis in *Drosophila* disrupts the Wingless, Hedgehog and Decapentaplegic signaling pathways. Development. 131**,** 1927-1938.

Bostwick, D. G., Burke, H. B., Djakiew, D., Euling, S., Ho, S. M., Landolph, J., Morrison, H., Sonawane, B., Shifflett, T., Waters, D. J., Timms, B., 2004. Human prostate cancer risk factors. Cancer. 101**,** 2371-2490.

Braat, A. K., Yan, N., Arn, E., Harrison, D., Macdonald, P. M., 2004. Localization-dependent oskar protein accumulation; control after the initiation of translation. Dev Cell. 7**,** 125-131.

Bravi, F., Scotti, L., Bosetti, C., Talamini, R., Negri, E., Montella, M., Franceschi, S., La Vecchia, C., 2006. Self-reported history of hypercholesterolaemia and gallstones and the risk of prostate cancer. Ann Oncol. 17**,** 1014-1017.

Brawer, M. K., Chetner, M. P., Beatie, J., Buchner, D. M., Vessella, R. L., Lange, P. H., 1992. Screening for prostatic carcinoma with prostate specific antigen. J Urol. 147**,** 841-845.

Brook, W. J., 2000. Hedgehog signaling and the axial patterning of *Drosophila* wings. Biochem Cell Biol. 78**,** 585-591.

Brumby, A. M., Richardson, H. E., 2005. Using *Drosophila melanogaster* to map human cancer pathways. Nat Rev Cancer. 5**,** 626-639.

Caldwell, M., Datta, S., 1998. Expression of cyclin E or DP/E2F rescues the G1 arrest of trol mutant neuroblasts in the *Drosophila* larval central nervous system. Mechanisms of Development. 79**,** 121-130.

Capdevila, J., Johnson, R. L., 2000. Hedgehog signaling in vertebrate and invertebrate limb patterning. Cell Mol Life Sci. 57**,** 1682-1694.

Chen, J. K., Taipale, J., Cooper, M. K., Beachy, P. A., 2002. Inhibition of Hedgehog signaling by direct binding of cyclopamine to Smoothened. Genes Dev. 16**,** 2743-2748.

Cohen, I. R., Murdoch, A. D., Naso, M. F., Marchetti, D., Berd, D., Iozzo, R. V., 1994. Abnormal expression of perlecan proteoglycan in metastatic melanomas. Cancer Res. 54**,** 5771-5774.

Collier, L. S., Largaespada, D. A., 2006. Transforming science: cancer gene identification. Curr Opin Genet Dev. 16**,** 23-29.

Conlon, E. M., Goode, E. L., Gibbs, M., Stanford, J. L., Badzioch, M., Janer, M., Kolb, S., Hood, L., Ostrander, E. A., Jarvik, G. P., Wijsman, E. M., 2003. Oligogenic segregation analysis of hereditary prostate cancer pedigrees: evidence for multiple loci affecting age at onset. Int J Cancer. 105**,** 630-635.

Cooper, M. K., Wassif, C. A., Krakowiak, P. A., Taipale, J., Gong, R., Kelley, R. I., Porter, F. D., Beachy, P. A., 2003. A defective response to Hedgehog signaling in disorders of cholesterol biosynthesis. Nat Genet. 33**,** 508-513.

Cronauer, M. V., Schulz, W. A., Seifert, H. H., Ackermann, R., Burchardt, M., 2003. Fibroblast growth factors and their receptors in urological cancers: basic research and clinical implications. Eur Urol. 43**,** 309-319.

D'Amico, A. V., Whittington, R., Malkowicz, S. B., Renshaw, A. A., Tomaszewski, J. E., Bentley, C., Schultz, D., Rocha, S., Wein, A., Richie, J. P., 2001. Estimating the impact on prostate cancer mortality of incorporating prostate-specific antigen testing into screening. Urology. 58**,** 406-410.

Dahmane, N., Lee, J., Robins, P., Heller, P., Ruiz i Altaba, A., 1997. Activation of the transcription factor Gli1 and the Sonic hedgehog signalling pathway in skin tumours. Nature. 389**,** 876-881.

Dahmane, N., Sanchez, P., Gitton, Y., Palma, V., Sun, T., Beyna, M., Weiner, H., Ruiz i Altaba, A., 2001. The Sonic Hedgehog-Gli pathway regulates dorsal brain growth and tumorigenesis. Development. 128**,** 5201-5212.

Datta, M. W., Hernandez, A. M., Schlicht, M. J., Kahler, A. J., Degueme, A. M., Dhir, R., Shah, R. B., Farach-Carson, M. C., Barrett, A., Datta, S., 2006a. Perlecan, a candidate gene for the CABP locus, regulates prostate cancer cell growth via the Sonic Hedgehog pathway. Mol Cancer. 5**,** 9-24.

Datta, S., 1995. Control of proliferation activation in quiescent neuroblasts of the *Drosophila* central nervous system. Development. 121**,** 1173-1182.

Datta, S., 1999. Activation of neuroblast proliferation in explant culture of the *Drosophila* larval CNS. Brain Research. 818**,** 77-83.

Datta, S., Datta, M. W., 2006. Sonic Hedgehog signaling in advanced prostate cancer. Cell Mol Life Sci. 63,435-448

Datta, S., Pierce, M., Datta, M. W., 2006b. Perlecan signaling: helping hedgehog stimulate prostate cancer growth. Int J Biochem Cell Biol. 38**,** 1855-1861.

Datta, S. 2007. HhN inhibits neuroblast proliferation in a dose- dependent manner in the *Drosophila* larval brain. Unpublished report, Datta lab, Texas A&M University, College Station.

Dawber, R. J., Hebbes, S., Herpers, B., Docquier, F., van den Heuvel, M., 2005. Differential range and activity of various forms of the Hedgehog protein. BMC Dev Biol. 5**,** 21-34.

De Marzo, A. M., Nelson, W. G., Meeker, A. K., Coffey, D. S., 1998. Stem cell features of benign and malignant prostate epithelial cells. J Urol. 160**,** 2381-2392.

De Marzo, A. M., Platz, E. A., Sutcliffe, S., Xu, J., Gronberg, H., Drake, C. G., Nakai, Y., Isaacs, W. B., Nelson, W. G., 2007. Inflammation in prostate carcinogenesis. Nat Rev Cancer. 7**,** 256-269.

DeMarzo, A. M., Nelson, W. G., Isaacs, W. B., Epstein, J. I., 2003. Pathological and molecular aspects of prostate cancer. Lancet. 361**,** 955-964.

Desbordes, S. C., Sanson, B., 2003. The glypican Dally-like is required for Hedgehog signalling in the embryonic epidermis of *Drosophila*. Development. 130**,** 6245-6255.

Dillin, A., Crawford, D. K., Kenyon, C., 2002. Timing requirements for insulin/IGF-1 signaling in *C. elegans*. Science. 298**,** 830-834.

Dong, J., Gailani, M. R., Pomeroy, S. L., Reardon, D., Bale, A. E., 2000. Identification of PATCHED mutations in medulloblastomas by direct sequencing. Hum Mutat. 16**,** 89-90.

Easton, D. F., Schaid, D. J., Whittemore, A. S., Isaacs, W. J., 2003. Where are the prostate cancer genes?-A summary of eight genome wide searches. Prostate. 57**,** 261-269.

Eaton, S., 2006. Release and trafficking of lipid-linked morphogens. Curr Opin Genet Dev. 16**,** 17-22.

Echelard, Y., Epstein, D. J., St-Jacques, B., Shen, L., Mohler, J., McMahon, J. A., McMahon, A. P., 1993. Sonic hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity. Cell. 75**,** 1417-1430.

Ephrussi, A., Lehmann, R., 1992. Induction of germ cell formation by oskar. Nature. 358**,** 387-392.

Ericson, J., Morton, S., Kawakami, A., Roelink, H., Jessell, T. M., 1996. Two critical periods of Sonic Hedgehog signaling required for the specification of motor neuron identity. Cell. 87**,** 661-673.

Fan, L., Pepicelli, C. V., Dibble, C. C., Catbagan, W., Zarycki, J. L., Laciak, R., Gipp, J., Shaw, A., Lamm, M. L., Munoz, A., Lipinski, R., Thrasher, J. B., Bushman, W., 2004. Hedgehog signaling promotes prostate xenograft tumor growth. Endocrinology. 145**,** 3961-3970.

Feldman, B. J., Feldman, D., 2001. The development of androgen-independent prostate cancer. Nat Rev Cancer. 1**,** 34-45.

Feng, S., Wang, F., Matsubara, A., Kan, M., McKeehan, W. L., 1997. Fibroblast growth factor receptor 2 limits and receptor 1 accelerates tumorigenicity of prostate epithelial cells. Cancer Res. 57**,** 5369-5378.

Flores, G. V., Duan, H., Yan, H., Nagaraj, R., Fu, W., Zou, Y., Noll, M., Banerjee, U., 2000. Combinatorial signaling in the specification of unique cell fates. Cell. 103**,** 75-85.

Freestone, S. H., Marker, P., Grace, O. C., Tomlinson, D. C., Cunha, G. R., Harnden, P., Thomson, A. A., 2003. Sonic hedgehog regulates prostatic growth and epithelial differentiation. Dev Biol. 264**,** 352-362.

Fuster, M. M., Esko, J. D., 2005. The sweet and sour of cancer: glycans as novel therapeutic targets. Nat Rev Cancer. 5**,** 526-542.

Gallet, A., Ruel, L., Staccini-Lavenant, L., Therond, P. P., 2006. Cholesterol modification is necessary for controlled planar long-range activity of Hedgehog in *Drosophila* epithelia. Development. 133**,** 407-418.

Gelmann, E. P., 2008. Complexities of Prostate-Cancer Risk. N Engl J Med. 358**,** 961-963.

Gibbs, M., Stanford, J. L., McIndoe, R. A., Jarvik, G. P., Kolb, S., Goode, E. L., Chakrabarti, L., Schuster, E. F., Buckley, V. A., Miller, E. L., Brandzel, S., Li, S., Hood, L., Ostrander, E. A., 1999. Evidence for a rare prostate cancer-susceptibility locus at chromosome 1p36. Am J Hum Genet. 64**,** 776-787.

Goodrich, L. V., Johnson, R. L., Milenkovic, L., McMahon, J. A., Scott, M. P., 1996. Conservation of the hedgehog/patched signaling pathway from flies to mice: induction of a mouse patched gene by Hedgehog. Genes Dev. 10**,** 301-312.

Grachtchouk, V., Grachtchouk, M., Lowe, L., Johnson, T., Wei, L., Wang, A., de Sauvage, F., Dlugosz, A. A., 2003. The magnitude of hedgehog signaling activity defines skin tumor phenotype. Embo J. 22**,** 2741-2751.

Greene, M. H., 1999. The genetics of hereditary melanoma and nevi. 1998 update. Cancer. 86**,** 2464-2477.

Guerrero, I., Chiang, C., 2007. A conserved mechanism of Hedgehog gradient formation by lipid modifications. Trends Cell Biol. 17**,** 1-5.

Hahn, H., Wicking, C., Zaphiropoulous, P. G., Gailani, M. R., Shanley, S., Chidambaram, A., Vorechovsky, I., Holmberg, E., Unden, A. B., Gillies, S., Negus, K., Smyth, I., Pressman, C., Leffell, D. J., Gerrard, B., Goldstein, A. M., Dean, M., Toftgard, R., Chenevix-Trench, G., Wainwright, B., Bale, A. E., 1996. Mutations of the human homolog of *Drosophila* patched in the nevoid basal cell carcinoma syndrome. Cell. 85**,** 841-851.

Haigis, M. C., Guarente, L. P., 2006. Mammalian sirtuins--emerging roles in physiology, aging, and calorie restriction. Genes Dev. 20**,** 2913-2921.

Han, C., Belenkaya, T. Y., Wang, B., Lin, X., 2004. *Drosophila* glypicans control the cell-to-cell movement of Hedgehog by a dynamin-independent process. Development. 131**,** 601-611.

Hassell, J., Yamada, Y., Arikawa-Hirasawa, E., 2002. Role of perlecan in skeletal development and diseases. Glycoconj J. 19**,** 263-267.

Hecht, J. T., Hall, C. R., Snuggs, M., Hayes, E., Haynes, R., Cole, W. G., 2002. Heparan sulfate abnormalities in exostosis growth plates. Bone. 31**,** 199-204.

Helfand, S. L., Rogina, B., 2003. Genetics of aging in the fruit fly, *Drosophila melanogaster*. Annu Rev Genet. 37**,** 329-348.

Hemmati, H. D., Nakano, I., Lazareff, J. A., Masterman-Smith, M., Geschwind, D. H., Bronner-Fraser, M., Kornblum, H. I., 2003. Cancerous stem cells can arise from pediatric brain tumors. Proc Natl Acad Sci U S A. 100**,** 15178-15183.

Hirayama, R., Takemura, K., Nihei, Z., Ichikawa, W., Takagi, Y., Mishima, Y., Utsuyama, M., Hirokawa, K., 1993. Differential effect of host microenvironment and systemic humoral factors on the implantation and the growth rate of metastatic tumor in parabiotic mice constructed between young and old mice. Mech Ageing Dev. 71**,** 213-221.

Hoque, A., Chen, H., Xu, X. C., 2008. Statin induces apoptosis and cell growth arrest in prostate cancer cells. Cancer Epidemiol Biomarkers Prev. 17**,** 88-94.

Horoszewicz, J. S., Leong, S. S., Chu, T. M., Wajsman, Z. L., Friedman, M., Papsidero, L., Kim, U., Chai, L. S., Kakati, S., Arya, S. K., Sandberg, A. A., 1980. The LNCaP cell line--a new model for studies on human prostatic carcinoma. Prog Clin Biol Res. 37**,** 115-132.

Huber, M. A., Kraut, N., Beug, H., 2005. Molecular requirements for epithelial-mesenchymal transition during tumor progression. Curr Opin Cell Biol. 17**,** 548-558.

Humphrey, P. A., 2007. Diagnosis of adenocarcinoma in prostate needle biopsy tissue. J Clin Pathol. 60**,** 35-42.

Huntly, B. J., Gilliland, D. G., 2005. Leukaemia stem cells and the evolution of cancer-stem-cell research. Nat Rev Cancer. 5**,** 311-321.

Huntly, B. J., Shigematsu, H., Deguchi, K., Lee, B. H., Mizuno, S., Duclos, N., Rowan, R., Amaral, S., Curley, D., Williams, I. R., Akashi, K., Gilliland, D. G., 2004. MOZ-TIF2, but not BCR-ABL, confers properties of leukemic stem cells to committed murine hematopoietic progenitors. Cancer Cell. 6**,** 587-596.

Hynes, M., Stone, D. M., Dowd, M., Pitts-Meek, S., Goddard, A., Gurney, A., Rosenthal, A., 1997. Control of cell pattern in the neural tube by the zinc finger transcription factor and oncogene Gli-1. Neuron. 19**,** 15-26.

Ingham, P. W., McMahon, A. P., 2001. Hedgehog signaling in animal development: paradigms and principles. Genes Dev. 15**,** 3059-3087.

Iozzo, R. V., Cohen, I. R., Grassel, S., Murdoch, A. D., 1994. The biology of perlecan: the multifaceted heparan sulphate proteoglycan of basement membranes and pericellular matrices. Biochem J. 302**,** 625-639.

Janer, M., Friedrichsen, D. M., Stanford, J. L., Badzioch, M. D., Kolb, S., Deutsch, K., Peters, M. A., Goode, E. L., Welti, R., DeFrance, H. B., Iwasaki, L., Li, S., Hood, L., Ostrander, E. A., Jarvik, G. P., 2003. Genomic scan of 254 hereditary prostate cancer families. Prostate. 57**,** 309-319.

Johnson, R. L., Rothman, A. L., Xie, J., Goodrich, L. V., Bare, J. W., Bonifas, J. M., Quinn, A. G., Myers, R. M., Cox, D. R., Epstein, E. H., Jr., Scott, M. P., 1996. Human homolog of patched, a candidate gene for the basal cell nevus syndrome. Science. 272**,** 1668-1671.

Kaighn, M. E., Lechner, J. F., Narayan, K. S., Jones, L. W., 1978. Prostate carcinoma: tissue culture cell lines. Natl Cancer Inst Monogr. 17-21.

Kaplan-Lefko, P. J., Chen, T. M., Ittmann, M. M., Barrios, R. J., Ayala, G. E., Huss, W. J., Maddison, L. A., Foster, B. A., Greenberg, N. M., 2003. Pathobiology of autochthonous prostate cancer in a pre-clinical transgenic mouse model. Prostate. 55**,** 219-237.

Karhadkar, S. S., Bova, G. S., Abdallah, N., Dhara, S., Gardner, D., Maitra, A., Isaacs, J. T., Berman, D. M., Beachy, P. A., 2004. Hedgehog signalling in prostate regeneration, neoplasia and metastasis. Nature. 431**,** 707-712.

Karpen, H. E., Bukowski, J. T., Hughes, T., Gratton, J. P., Sessa, W. C., Gailani, M. R., 2001. The sonic hedgehog receptor patched associates with caveolin-1 in cholesterol-rich microdomains of the plasma membrane. J Biol Chem. 276**,** 19503-19511.

Katayam, M., Yoshida, K., Ishimori, H., Katayama, M., Kawase, T., Motoyama, J., Kamiguchi, H., 2002. Patched and smoothened mRNA expression in human astrocytic tumors inversely correlates with histological malignancy. J Neurooncol. 59**,** 107-115.

Kayed, H., Kleeff, J., Keleg, S., Buchler, M. W., Friess, H., 2003. Distribution of Indian hedgehog and its receptors patched and smoothened in human chronic pancreatitis. J Endocrinol. 178**,** 467-478.

Kenyon, C., 2001. A conserved regulatory system for aging. Cell. 105**,** 165-168.

Kipling, D., Davis, T., Ostler, E. L., Faragher, R. G., 2004. What can progeroid syndromes tell us about human aging? Science. 305**,** 1426-1431.

Klebes, A., Biehs, B., Cifuentes, F., Kornberg, T. B., 2002. Expression profiling of *Drosophila* imaginal discs. Genome Biol. 3**,** 1-16.

Kopnin, B. P., 2000. Targets of oncogenes and tumor suppressors: key for understanding basic mechanisms of carcinogenesis. Biochemistry (Mosc). 65**,** 2-27.

Kristal, A. R., Chi, C., Tangen, C. M., Goodman, P. J., Etzioni, R., Thompson, I. M., 2006. Associations of demographic and lifestyle characteristics with prostate-specific antigen (PSA) concentration and rate of PSA increase. Cancer. 106**,** 320-328.

Krtolica, A., Parrinello, S., Lockett, S., Desprez, P. Y., Campisi, J., 2001. Senescent fibroblasts promote epithelial cell growth and tumorigenesis: a link between cancer and aging. Proc Natl Acad Sci U S A. 98**,** 12072-12077.

Kuefer, R., Varambally, S., Zhou, M., Lucas, P. C., Loeffler, M., Wolter, H., Mattfeldt, T., Hautmann, R. E., Gschwend, J. E., Barrette, T. R., Dunn, R. L., Chinnaiyan, A. M., Rubin, M. A., 2002. alpha-Methylacyl-CoA racemase: expression levels of this novel cancer biomarker depend on tumor differentiation. Am J Pathol. 161**,** 841-848.

Kumar, S., Balczarek, K. A., Lai, Z. C., 1996. Evolution of the hedgehog gene family. Genetics. 142**,** 965-972.

Kwabi-Addo, B., Wang, J., Erdem, H., Vaid, A., Castro, P., Ayala, G., Ittmann, M., 2004. The expression of Sprouty1, an inhibitor of fibroblast growth factor signal transduction, is decreased in human prostate cancer. Cancer Res. 64**,** 4728-4735.

Lai, J., Chien, J., Staub, J., Avula, R., Greene, E. L., Matthews, T. A., Smith, D. I., Kaufmann, S. H., Roberts, L. R., Shridhar, V., 2003a. Loss of HSulf-1 up-regulates heparin-binding growth factor signaling in cancer. J Biol Chem. 278**,** 23107-23117.

Lai, K., Kaspar, B. K., Gage, F. H., Schaffer, D. V., 2003b. Sonic hedgehog regulates adult neural progenitor proliferation *in vitro* and *in vivo*. Nat Neurosci. 6**,** 21-27.

Lamm, M. L., Catbagan, W. S., Laciak, R. J., Barnett, D. H., Hebner, C. M., Gaffield, W., Walterhouse, D., Iannaccone, P., Bushman, W., 2002. Sonic hedgehog activates mesenchymal Gli1 expression during prostate ductal bud formation. Dev Biol. 249**,** 349-366.

Lander, A. D., 2007. Morpheus unbound: reimagining the morphogen gradient. Cell. 128**,** 245-256.

Lee, J., Platt, K. A., Censullo, P., Ruiz i Altaba, A., 1997. Gli1 is a target of Sonic hedgehog that induces ventral neural tube development. Development. 124**,** 2537-2552.

Li, Y., Welm, B., Podsypanina, K., Huang, S., Chamorro, M., Zhang, X., Rowlands, T., Egeblad, M., Cowin, P., Werb, Z., Tan, L. K., Rosen, J. M., Varmus, H. E., 2003. Evidence that transgenes encoding components of the Wnt signaling pathway preferentially induce mammary cancers from progenitor cells. Proc Natl Acad Sci U S A. 100**,** 15853-15858.

Lin, X., 2004. Functions of heparan sulfate proteoglycans in cell signaling during development. Development. 131**,** 6009-6021.

Lin, X., Buff, E. M., Perrimon, N., Michelson, A. M., 1999. Heparan sulfate proteoglycans are essential for FGF receptor signaling during *Drosophila* embryonic development. Development. 126**,** 3715-3723.

Lindholm, P. F., Bub, J., Kaul, S., Shidham, V. B., Kajdacsy-Balla, A., 2000. The role of constitutive NF-kappaB activity in PC-3 human prostate cancer cell invasive behavior. Clin Exp Metastasis. 18**,** 471-479.

Liu, A. Y., True, L. D., 2002. Characterization of prostate cell types by CD cell surface molecules. Am J Pathol. 160**,** 37-43.

Llorente, A., van Deurs, B., Sandvig, K., 2007. Cholesterol regulates prostasome release from secretory lysosomes in PC-3 human prostate cancer cells. Eur J Cell Biol. 86**,** 405-415.

Lum, L., Yao, S., Mozer, B., Rovescalli, A., Von Kessler, D., Nirenberg, M., Beachy, P. A., 2003. Identification of Hedgehog pathway components by RNAi in *Drosophila* cultured cells. Science. 299**,** 2039-2045.

Lung, O., Wolfner, M. F., 2001. Identification and characterization of the major *Drosophila melanogaster* mating plug protein. Insect Biochem Mol Biol. 31**,** 543-551.

Ma, X., Chen, K., Huang, S., Zhang, X., Adegboyega, P. A., Evers, B. M., Zhang, H., Xie, J., 2005. Frequent activation of the hedgehog pathway in advanced gastric adenocarcinomas. Carcinogenesis. 26**,** 1698-1705.

Ma, X., Sheng, T., Zhang, Y., Zhang, X., He, J., Huang, S., Chen, K., Sultz, J., Adegboyega, P. A., Zhang, H., Xie, J., 2006. Hedgehog signaling is activated in subsets of esophageal cancers. Int J Cancer. 118**,** 139-148.

Machold, R., Hayashi, S., Rutlin, M., Muzumdar, M. D., Nery, S., Corbin, J. G., Gritli-Linde, A., Dellovade, T., Porter, J. A., Rubin, L. L., Dudek, H., McMahon, A. P., Fishell, G., 2003. Sonic hedgehog is required for progenitor cell maintenance in telencephalic stem cell niches. Neuron. 39**,** 937-950.

Mann, R. K., Beachy, P. A., 2000. Cholesterol modification of proteins. Biochim Biophys Acta. 1529**,** 188-202.

Marcelle, C., Stark, M. R., Bronner-Fraser, M., 1997. Coordinate actions of BMPs, Wnts, Shh and noggin mediate patterning of the dorsal somite. Development. 124**,** 3955-3963.

Martel, C. L., Gumerlock, P. H., Meyers, F. J., Lara, P. N., 2003. Current strategies in the management of hormone refractory prostate cancer. Cancer Treat Rev. 29**,** 171-187.

Matysiak, B. E., Brodzeller, T., Buck, S., French, A., Counts, C., Boorsma, B., Datta, M. W., Kajdacsy-Balla, A. A., 2003. Simple, inexpensive method for automating tissue microarray production provides enhanced microarray reproducibility. Appl Immunohistochem Mol Morphol. 11**,** 269-273.

McCullough, K. D., Coleman, W. B., Smith, G. J., Grisham, J. W., 1997. Age-dependent induction of hepatic tumor regression by the tissue microenvironment after transplantation of neoplastically transformed rat liver epithelial cells into the liver. Cancer Res. 57**,** 1807-1813.

Mehra, R., 2007. Global public health problem of sudden cardiac death. J Electrocardiol. 40**,** S118-122.

Miquel, J., Lundgren, P. R., Bensch, K. G., Atlan, H., 1976. Effects of temperature on the life span, vitality and fine structure of *Drosophila melanogaster*. Mech Ageing Dev. 5**,** 347-370.

Nackaerts, K., Verbeken, E., Deneffe, G., Vanderschueren, B., Demedts, M., David, G., 1997. Heparan sulfate proteoglycan expression in human lung-cancer cells. Int J Cancer. 74**,** 335-345.

Nelson, C. M., Bissell, M. J., 2006. Of extracellular matrix, scaffolds, and signaling: tissue architecture regulates development, homeostasis, and cancer. Annu Rev Cell Dev Biol. 22**,** 287-309.

Nelson, W. G., De Marzo, A. M., Isaacs, W. B., 2003. Prostate cancer. N Engl J Med. 349**,** 366-381.

Nusslein-Volhard, C., Wieschaus, E., 1980. Mutations affecting segment number and polarity in *Drosophila*. Nature. 287**,** 795-801.

Nybakken, K., Perrimon, N., 2002a. Hedgehog signal transduction: recent findings. Curr Opin Genet Dev. 12**,** 503-511.

Nybakken, K., Perrimon, N., 2002b. Heparan sulfate proteoglycan modulation of developmental signaling in *Drosophila*. Biochim Biophys Acta. 1573**,** 280-291.

Pagliarini, R. A., Xu, T., 2003. A genetic screen in *Drosophila* for metastatic behavior. Science. 302**,** 1227-1231.

Palma, V., Lim, D. A., Dahmane, N., Sanchez, P., Brionne, T. C., Herzberg, C. D., Gitton, Y., Carleton, A., Alvarez-Buylla, A., Ruiz i Altaba, A., 2005. Sonic hedgehog controls stem cell behavior in the postnatal and adult brain. Development. 132**,** 335-344.

Palma, V., Ruiz i Altaba, A., 2004. Hedgehog-GLI signaling regulates the behavior of cells with stem cell properties in the developing neocortex. Development. 131**,** 337-345.

Panakova, D., Sprong, H., Marois, E., Thiele, C., Eaton, S., 2005. Lipoprotein particles are required for Hedgehog and Wingless signalling. Nature. 435**,** 58-65.

Pardal, R., Clarke, M. F., Morrison, S. J., 2003. Applying the principles of stem-cell biology to cancer. Nat Rev Cancer. 3**,** 895-902.

Parisi, M. J., Lin, H., 1998. The role of the hedgehog/patched signaling pathway in epithelial stem cell proliferation: from fly to human. Cell Res. 8**,** 15-21.

Park, C. H., Bergsagel, D. E., McCulloch, E. A., 1971. Mouse myeloma tumor stem cells: a primary cell culture assay. J Natl Cancer Inst. 46**,** 411-422.

Park, H. L., Bai, C., Platt, K. A., Matise, M. P., Beeghly, A., Hui, C. C., Nakashima, M., Joyner, A. L., 2000. Mouse Gli1 mutants are viable but have defects in SHH signaling in combination with a Gli2 mutation. Development. 127**,** 1593-1605.

Park, Y., Fujioka, M., Kobayashi, M., Jaynes, J. B., Datta, S., 2001. even skipped is required to produce a trans-acting signal for larval neuroblast proliferation that can be mimicked by ecdysone. Development. 128**,** 1899-1909.

Park, Y., Rangel, C., Reynolds, M. M., Caldwell, M. C., Johns, M., Nayak, M., Welsh, C. J., McDermott, S., Datta, S., 2003. *Drosophila* perlecan modulates FGF and hedgehog signals to activate neural stem cell division. Dev Biol. 253**,** 247-257.

Pasca di Magliano, M., Hebrok, M., 2003. Hedgehog signalling in cancer formation and maintenance. Nat Rev Cancer. 3**,** 903-911.

Pepinsky, R. B., Zeng, C., Wen, D., Rayhorn, P., Baker, D. P., Williams, K. P., Bixler, S. A., Ambrose, C. M., Garber, E. A., Miatkowski, K., Taylor, F. R., Wang, E. A., Galdes, A., 1998. Identification of a palmitic acid-modified form of human Sonic hedgehog. J Biol Chem. 273**,** 14037-14045.

Pettaway, C. A., Pathak, S., Greene, G., Ramirez, E., Wilson, M. R., Killion, J. J., Fidler, I. J., 1996. Selection of highly metastatic variants of different human prostatic carcinomas using orthotopic implantation in nude mice. Clin Cancer Res. 2**,** 1627-1636.

Pietsch, T., Waha, A., Koch, A., Kraus, J., Albrecht, S., Tonn, J., Sorensen, N., Berthold, F., Henk, B., Schmandt, N., Wolf, H. K., von Deimling, A., Wainwright, B., Chenevix-Trench, G., Wiestler, O. D., Wicking, C., 1997. Medulloblastomas of the desmoplastic variant carry mutations of the human homologue of *Drosophila* patched. Cancer Res. 57**,** 2085-2088.

Platz, E., Leitzmann, M., Visvanathan, K., Stampfer, M., Willett, W., Rimm, E., Giovannucci, E. 2005. Cholesterol-Lowering Drugs including Statins and Risk of Prostate Cancer in a Large Prospective Cohort Study., American Association of Cancer Research, Anaheim, California.

Podlasek, C. A., Barnett, D. H., Clemens, J. Q., Bak, P. M., Bushman, W., 1999. Prostate development requires Sonic hedgehog expressed by the urogenital sinus epithelium. Dev Biol. 209**,** 28-39.

Pomeroy, S. L., Tamayo, P., Gaasenbeek, M., Sturla, L. M., Angelo, M., McLaughlin, M. E., Kim, J. Y., Goumnerova, L. C., Black, P. M., Lau, C., Allen, J. C., Zagzag, D., Olson, J. M., Curran, T., Wetmore, C., Biegel, J. A., Poggio, T., Mukherjee, S., Rifkin, R., Califano, A., Stolovitzky, G., Louis, D. N., Mesirov, J. P., Lander, E. S., Golub, T. R., 2002. Prediction of central nervous system embryonal tumour outcome based on gene expression. Nature. 415**,** 436-442.

Porter, J. A., Ekker, S. C., Park, W. J., von Kessler, D. P., Young, K. E., Chen, C. H., Ma, Y., Woods, A. S., Cotter, R. J., Koonin, E. V., Beachy, P. A., 1996a. Hedgehog patterning activity: role of a lipophilic modification mediated by the carboxy-terminal autoprocessing domain. Cell. 86**,** 21-34.

Porter, J. A., von Kessler, D. P., Ekker, S. C., Young, K. E., Lee, J. J., Moses, K., Beachy, P. A., 1995. The product of hedgehog autoproteolytic cleavage active in local and long-range signalling. Nature. 374**,** 363-366.

Porter, J. A., Young, K. E., Beachy, P. A., 1996b. Cholesterol modification of hedgehog signaling proteins in animal development. Science. 274**,** 255-259.

Potter, J. D., 2007. Morphogens, morphostats, microarchitecture and malignancy. Nat Rev Cancer. 7**,** 464-474.

Pugh, T. D., Chang, C., Uemura, H., Weindruch, R., 1994. Prostatic localization of spontaneous early invasive carcinoma in Lobund-Wistar rats. Cancer Res. 54**,** 5766-5770.

Raffel, C., Jenkins, R. B., Frederick, L., Hebrink, D., Alderete, B., Fults, D. W., James, C. D., 1997. Sporadic medulloblastomas contain PTCH mutations. Cancer Res. 57**,** 842-845.

Rapraeger, A. C., Krufka, A., Olwin, B. B., 1991. Requirement of heparan sulfate for bFGF-mediated fibroblast growth and myoblast differentiation. Science. 252**,** 1705-1708.

Regl, G., Neill, G. W., Eichberger, T., Kasper, M., Ikram, M. S., Koller, J., Hintner, H., Quinn, A. G., Frischauf, A. M., Aberger, F., 2002. Human GLI2 and GLI1 are part of a positive feedback mechanism in Basal Cell Carcinoma. Oncogene. 21**,** 5529-5539.

Reifenberger, J., Wolter, M., Weber, R. G., Megahed, M., Ruzicka, T., Lichter, P., Reifenberger, G., 1998. Missense mutations in SMOH in sporadic basal cell carcinomas of the skin and primitive neuroectodermal tumors of the central nervous system. Cancer Res. 58**,** 1798-1803.

Reya, T., Morrison, S. J., Clarke, M. F., Weissman, I. L., 2001. Stem cells, cancer, and cancer stem cells. Nature. 414**,** 105-111.

Rhim, J. S., 2000. *In vitro* human cell culture models for the study of prostate cancer. Prostate Cancer Prostatic Dis. 3**,** 229-235.

Roessler, E., Du, Y. Z., Mullor, J. L., Casas, E., Allen, W. P., Gillessen-Kaesbach, G., Roeder, E. R., Ming, J. E., Ruiz i Altaba, A., Muenke, M., 2003. Loss-of-function mutations in the human GLI2 gene are associated with pituitary anomalies and holoprosencephaly-like features. Proc Natl Acad Sci U S A. 100**,** 13424-13429.

Rogina, B., Reenan, R. A., Nilsen, S. P., Helfand, S. L., 2000. Extended life-span conferred by cotransporter gene mutations in *Drosophila*. Science. 290**,** 2137-2140.

Rubin, J. B., Choi, Y., Segal, R. A., 2002. Cerebellar proteoglycans regulate sonic hedgehog responses during development. Development. 129**,** 2223-2232.

Ruiz i Altaba, A., Palma, V., Dahmane, N., 2002. Hedgehog-Gli signalling and the growth of the brain. Nat Rev Neurosci. 3**,** 24-33.

Ruiz i Altaba, A., Stecca, B., Sanchez, P., 2004. Hedgehog--Gli signaling in brain tumors: stem cells and paradevelopmental programs in cancer. Cancer Lett. 204**,** 145-157.

Sanchez, P., Hernandez, A. M., Stecca, B., Kahler, A. J., DeGueme, A. M., Barrett, A., Beyna, M., Datta, M. W., Datta, S., Ruiz i Altaba, A., 2004. Inhibition of prostate cancer proliferation by interference with SONIC HEDGEHOG-GLI1 signaling. Proc Natl Acad Sci U S A. 101**,** 12561-12566.

Sancho, E., Batlle, E., Clevers, H., 2004. Signaling pathways in intestinal development and cancer. Annu Rev Cell Dev Biol. 20**,** 695-723.

Savore, C., Zhang, C., Muir, C., Liu, R., Wyrwa, J., Shu, J., Zhau, H. E., Chung, L. W., Carson, D. D., Farach-Carson, M. C., 2005. Perlecan knockdown in metastatic prostate cancer cells reduces heparin-binding growth factor responses in vitro and tumor growth in vivo. Clin Exp Metastasis. 22**,** 377-390.

Schlicht, M., Matysiak, B., Brodzeller, T., Wen, X., Liu, H., Zhou, G., Dhir, R., Hessner, M. J., Tonellato, P., Suckow, M., Pollard, M., Datta, M. W., 2004. Cross-species global and subset gene expression profiling identifies genes involved in prostate cancer response to selenium. BMC Genomics. 5**,** 58-69.

SEER Registry Database, http://seer.cancer.gov. Accessed February 2008

Shah, R. B., Mehra, R., Chinnaiyan, A. M., Shen, R., Ghosh, D., Zhou, M., Macvicar, G. R., Varambally, S., Harwood, J., Bismar, T. A., Kim, R., Rubin, M. A., Pienta, K. J., 2004. Androgen-independent prostate cancer is a heterogeneous group of diseases: lessons from a rapid autopsy program. Cancer Res. 64**,** 9209-9216.

Sharma, B., Handler, M., Eichstetter, I., Whitelock, J. M., Nugent, M. A., Iozzo, R. V., 1998. Antisense targeting of perlecan blocks tumor growth and angiogenesis *in vivo*. J Clin Invest. 102**,** 1599-1608.

Sheng, T., Li, C., Zhang, X., Chi, S., He, N., Chen, K., McCormick, F., Gatalica, Z., Xie, J., 2004. Activation of the hedgehog pathway in advanced prostate cancer. Mol Cancer. 3**,** 29-42.

Spring, J., Paine-Saunders, S. E., Hynes, R. O., Bernfield, M., 1994. *Drosophila* syndecan: conservation of a cell-surface heparan sulfate proteoglycan. Proc Natl Acad Sci U S A. 91**,** 3334-3338.

Stein, U., Eder, C., Karsten, U., Haensch, W., Walther, W., Schlag, P. M., 1999. GLI gene expression in bone and soft tissue sarcomas of adult patients correlates with tumor grade. Cancer Res. 59**,** 1890-1895.

Stone, K. R., Mickey, D. D., Wunderli, H., Mickey, G. H., Paulson, D. F., 1978. Isolation of a human prostate carcinoma cell line (DU 145). Int J Cancer. 21**,** 274-281.

Su, V. F., Jones, K. A., Brodsky, M., The, I., 2007. Quantitative analysis of Hedgehog gradient formation using an inducible expression system. BMC Dev Biol. 7**,** 43-58.

Sutherland, D., Samakovlis, C., Krasnow, M. A., 1996. branchless encodes a *Drosophila* FGF homolog that controls tracheal cell migration and the pattern of branching. Cell. 87**,** 1091-1101.

Tabata, T., Kornberg, T. B., 1994. hedgehog is a signalling protein with a key role in patterning in *Drosophila*. Cell. 76**,** 89-102.

Taylor, M. D., Liu, L., Raffel, C., Hui, C. C., Mainprize, T. G., Zhang, X., Agatep, R., Chiappa, S., Gao, L., Lowrance, A., Hao, A., Goldstein, A. M., Stavrou, T., Scherer, S. W., Dura, W. T., Wainwright, B., Squire, J. A., Rutka, J. T., Hogg, D., 2002. Mutations in SUFU predispose to medulloblastoma. Nat Genet. 31**,** 306-310.

Thalmann, G. N., Sikes, R. A., Wu, T. T., Degeorges, A., Chang, S. M., Ozen, M., Pathak, S., Chung, L. W., 2000. LNCaP progression model of human prostate cancer: androgen-independence and osseous metastasis. Prostate. 44 (2)**,** 91-103, July.

Thayer, S. P., di Magliano, M. P., Heiser, P. W., Nielsen, C. M., Roberts, D. J., Lauwers, G. Y., Qi, Y. P., Gysin, S., Fernandez-del Castillo, C., Yajnik, V., Antoniu, B., McMahon, M., Warshaw, A. L., Hebrok, M., 2003. Hedgehog is an early and late mediator of pancreatic cancer tumorigenesis. Nature. 425**,** 851-856.

The, I., Bellaiche, Y., Perrimon, N., 1999. Hedgehog movement is regulated through tout velu-dependent synthesis of a heparan sulfate proteoglycan. Mol Cell. 4**,** 633-639.

Tkachenko, E., Rhodes, J. M., Simons, M., 2005. Syndecans: new kids on the signaling block. Circ Res. 96**,** 488-500.

Urbach, R., Schnabel, R., Technau, G. M., 2003. The pattern of neuroblast formation, mitotic domains and proneural gene expression during early brain development in *Drosophila*. Development. 130**,** 3589-3606.

Van der Horst, G., Farih-Sips, H., Lowik, C. W., Karperien, M., 2003. Hedgehog stimulates only osteoblastic differentiation of undifferentiated KS483 cells. Bone. 33**,** 899-910.

Volk, R., Schwartz, J. J., Li, J., Rosenberg, R. D., Simons, M., 1999. The role of syndecan cytoplasmic domain in basic fibroblast growth factor-dependent signal transduction. J Biol Chem. 274**,** 24417-24424.

Wakefield, L. M., Roberts, A. B., 2002. TGF-beta signaling: positive and negative effects on tumorigenesis. Curr Opin Genet Dev. 12**,** 22-29.

Wang, B. E., Shou, J., Ross, S., Koeppen, H., De Sauvage, F. J., Gao, W. Q., 2003. Inhibition of epithelial ductal branching in the prostate by sonic hedgehog is indirectly mediated by stromal cells. J Biol Chem. 278**,** 18506-18513.

Watkins, D. N., Berman, D. M., Burkholder, S. G., Wang, B., Beachy, P. A., Baylin, S. B., 2003. Hedgehog signalling within airway epithelial progenitors and in small-cell lung cancer. Nature. 422**,** 313-317.

Weiner, H. L., Bakst, R., Hurlbert, M. S., Ruggiero, J., Ahn, E., Lee, W. S., Stephen, D., Zagzag, D., Joyner, A. L., Turnbull, D. H., 2002. Induction of medulloblastomas in mice by sonic hedgehog, independent of Gli1. Cancer Res. 62**,** 6385-6389.

Wendler, F., Franch-Marro, X., Vincent, J. P., 2006. How does cholesterol affect the way Hedgehog works? Development. 133**,** 3055-3061.

Wheeler, D. L., Church, D. M., Lash, A. E., Leipe, D. D., Madden, T. L., Pontius, J. U., Schuler, G. D., Schriml, L. M., Tatusova, T. A., Wagner, L., Rapp, B. A., 2001. Database resources of the National Center for Biotechnology Information. Nucleic Acids Res. 29**,** 11-16.

Wolter, M., Reifenberger, J., Sommer, C., Ruzicka, T., Reifenberger, G., 1997. Mutations in the human homologue of the *Drosophila* segment polarity gene patched (PTCH) in sporadic basal cell carcinomas of the skin and primitive neuroectodermal tumors of the central nervous system. Cancer Res. 57**,** 2581-2585.

Woodhouse, E. C., Fisher, A., Bandle, R. W., Bryant-Greenwood, B., Charboneau, L., Petricoin, E. F., 3rd, Liotta, L. A., 2003. *Drosophila* screening model for metastasis: Semaphorin 5c is required for l(2)gl cancer phenotype. Proc Natl Acad Sci U S A. 100**,** 11463-11468.

Wu, H. C., Hsieh, J. T., Gleave, M. E., Brown, N. M., Pathak, S., Chung, L. W., 1994. Derivation of androgen-independent human LNCaP prostatic cancer cell sublines: role of bone stromal cells. Int J Cancer. 57**,** 406-412.

Wu, X., Kan, M., Wang, F., Jin, C., Yu, C., McKeehan, W. L., 2001. A rare premalignant prostate tumor epithelial cell syndecan-1 forms a fibroblast growth factor-binding complex with progression-promoting ectopic fibroblast growth factor receptor 1. Cancer Res. 61**,** 5295-5302.

Xu, J., Gillanders, E. M., Isaacs, S. D., Chang, B. L., Wiley, K. E., Zheng, S. L., Jones, M., Gildea, D., Riedesel, E., Albertus, J., Freas-Lutz, D., Markey, C., Meyers, D. A., Walsh, P. C., Trent, J. M., Isaacs, W. B., 2003. Genome-wide scan for prostate cancer susceptibility genes in the Johns Hopkins hereditary prostate cancer families. Prostate. 57**,** 320-325.

Zeng, X., Goetz, J. A., Suber, L. M., Scott, W. J., Jr., Schreiner, C. M., Robbins, D. J., 2001. A freely diffusible form of Sonic hedgehog mediates long-range signalling. Nature. 411**,** 716-720.

Zhang, J., Lipinski, R., Shaw, A., Gipp, J., Bushman, W., 2007. Lack of demonstrable autocrine hedgehog signaling in human prostate cancer cell lines. J Urol. 177**,** 1179-1185.

Zhang, Y., Kalderon, D., 2000. Regulation of cell proliferation and patterning in *Drosophila* oogenesis by Hedgehog signaling. Development. 127**,** 2165-2176.

Zhang, Y., Kalderon, D., 2001. Hedgehog acts as a somatic stem cell factor in the *Drosophila* ovary. Nature. 410**,** 599-604.

Zheng, S. L., Sun, J., Wiklund, F., Smith, S., Stattin, P., Li, G., Adami, H. O., Hsu, F. C., Zhu, Y., Balter, K., Kader, A. K., Turner, A. R., Liu, W., Bleecker, E. R., Meyers, D. A., Duggan, D., Carpten, J. D., Chang, B. L., Isaacs, W. B., Xu, J., Gronberg, H., 2008. Cumulative Association of Five Genetic Variants with Prostate Cancer. N Engl J Med. 358, 910-919.

Zhu, G., Mehler, M. F., Zhao, J., Yu Yung, S., Kessler, J. A., 1999. Sonic hedgehog and BMP2 exert opposing actions on proliferation and differentiation of embryonic neural progenitor cells. Dev Biol. 215**,** 118-129.

Zurawel, R. H., Allen, C., Chiappa, S., Cato, W., Biegel, J., Cogen, P., de Sauvage, F., Raffel, C., 2000. Analysis of PTCH/SMO/SHH pathway genes in medulloblastoma. Genes Chromosomes Cancer. 27**,** 44-51.

VITA

Name: Ana Maria Hernandez Cotes

Address: Department of Biochemistry and Biophysics, Texas A&M University, College Station, TX 77840-2128

Education: Ph.D. Biochemistry, Texas A&M University, College Station, 2008
B.S Microbiology, Universidad de Los Andes, Bogota, Colombia, 2001

Publications: Datta, M.W., Hernandez, A.M., Schlicht, M.J., Kahler, A.J., DeGueme A.M., Dhir, R., Shah, R.B., Farach-Carson, C., Barrett, A., and Datta, S. (2006). *PERLECAN*, a candidate gene for the CAPB locus, regulates prostate cancer growth via the SONIC HEDGEHOG pathway. Molecular Cancer, 5, 9-24.

Sanchez, P., Hernandez, A.M., Stecca, B., Kahler, A.J., DeGueme, A.M., Barrett, A., Beyna, M., Datta, M.W., Datta, S., and Ruiz i Altaba, A. (2004). Inhibition of prostate cancer proliferation by interference with SONIC HEDGEHOG-GLI1 signaling. Proc Natl Acad Sci U S A. 101, 12561-12566.