THE ROLE OF HNF4A IN GERM CELL TUMOR DEVELOPMENT

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DEDICATION

I dedicate this work to my family and friends who have kept me sane through these years.

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by

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Yolk sac tumor is a histological category of germ cell tumors, which represent the most frequent malignancy in young men. Little is known about the molecular mechanisms responsible for the aberrant differentiation and oncogenic potential of yolk sac tumors. Multiple recurrent chromosomal copy number variations are the primary genetic lesions discovered in yolk sac tumors. Frequent gains of chromosome 20q13 provide clues as to the identity of important YST driver genes. We have determined that the Hepatocyte Nuclear Factor 4 alpha locus, which resides in this region, is frequently amplified in YSTs. Overexpression of HNF4A in an undifferentiated GCT line is sufficient to drive extraembryonic endodermal gene programs. This endodermal program subsequently utilizes endogenous WNT signaling to grow. Additionally, isoform specific manipulation of HNF4A revealed differential oncogenic potential amongst HNF4A isoforms. This isoform specific manipulation was involved in modulation of the WNT

pathway which has previously been identified as active in YSTs. These results allowed us to uncover HNF4A isoform specific differences in histological samples from YST patients. Our findings reveal a possible new drug target in YST treatment and reveal an interesting isoform dependent mechanism for understanding tumor development.

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List of Abbreviations

GCT- Germ Cell Tumor

pGCT- Pediatric Germ Cell Tumor

aGCT- Adult Germ Cell Tumor

GCNIS- Germ Cell Neoplasia in situ

SGCT- Seminomatous Germ Cell Tumor

NSGCT- Non-Seminomatous Germ Cell Tumor

MMGCT- Mixed Malignant Germ Cell Tumor

EC- Embryonal Carcinoma

YST- Yolk Sac Tumor

TER- Teratoma

CC- Choriocarcinoma

AFP- Alpha Feto-Protein

CNV- Copy Number Variation

qRT-PCR- Quantitative Real Time Polymerase Chain Reaction

LBD- Ligand Binding Domain

HCC- Hepatocellular Carcinoma

CRC- Colorectal Cancer

TA- Transit Amplifying

ESC- Embryonic Stem Cell

iPS- Induced Pluripotent Stem Cell

huMSC- Human Umbilical Mesenchymal Stem Cell

TF- Transcription Factor

PGC- Primordial Germ Cell

PE- Primitive Endoderm

DE- Definitive Endoderm

GWAS- Genome Wide Association Study

SNP- Single Nucleotide Polymorphism

GR- Gonadal Ridge

EGGCT- Extragonadal Germ Cell Tumor

ANS- Autonomic Nervous System

RTK- Receptor Tyrosine Kinase

HRP- Horse Radish Peroxidase

ELISA- Enzyme-Linked Immunosorbent Assay

TMA- Tumor Microarray

IHC- Immunohistochemistry

IF- Immunofluorescence

Chapter I

Introduction to Germ Cell Tumors and HNF4A Based Endodermal Differentiation

Pediatric Cancer

The term Pediatric Cancer encompasses a wide variety of neoplastic malignancies found specifically in patients whose ages range from fetuses through adolescence and into young adulthood, typically ages 0-18 years. Though pediatric cancers occur at a relatively low rate in comparison to the incidence of adult tumors this class of malignancies present a unique set of challenges. As opposed to adult cancers in which onset and mortality present late in life, these tumors result in many more years of productive life lost in the case of mortality. Also, there is a significant incidence of physical and psychological adverse late effects of treatment in survivors or childhood cancer treatment. Cancer is the leading cause of disease related mortality in children ages 0-14. Though massive improvements in survival have been made in the previous decades they have generally been focused on using chemotherapeutics and radiological methods that function by killing rapidly dividing cells in the patient. This treatment schema, which is generally efficacious in the treatment of pediatric disease, is problematic in the pediatric patient whose body is full of cells rapidly dividing for the normal purposes of growth and development (Poynter, Amatruda, and Ross 2010; Williams et al. 1987). This “nonspecific” treatment modality is responsible for a myriad of late effects(Einhorn 2002). In adult men treated with

standard GCT chemotherapeutics late effects include, but are not limited to pulmonary fibrosis (Osanto et al. 1992), renal insufficiency and salt-wasting (Hansen et al. 1988), infertility and hormonal changes (Hansen and Hansen 1993; Hansen, Berthelsen, and von der Maase 1990; Berger et al. 1996), hyperlipidemia (Bissett et al. 1990), Raynaud's 8 phenomenon (Teutsch, Lipton, and Harvey 1977), obesity (Siviero-Miachon, Spinola-Castro, and Guerra-Junior 2009), and neuropathy (Glendenning et al.). Hearing loss through ototoxicity, as assessed by audiogram, is found in ~80% of patients (Strumberg et al. 2002). Chemotherapeutic treatment can result in twice the risk of early onset cardiovascular disease (Huddart et al. 2003)and second malignancies (Travis et al. 1997; Travis et al. 2005). This raises the important point that, although pediatric patients generally survive, these patients represent a vast amount of possible suffering over the course of a lifetime and further investigation into pediatric specific late effects is warranted. Additionally, relapse is still seen after cisplatin-based chemotherapy in 15% of all GCT patients and little is known about the specific effects on pediatric patients (Einhorn 2002). Therefore, pediatric cancer research likely represents an underrepresented section of the general basic science environment. Research into these diseases are still of vital importance as they benefit a small number of patients who previously had little hope and because the field of cancer biology is truly unified in that any knowledge about the agents and lesions driving malignancies can be useful to the field as a whole.

A primary focus of pediatric cancer today is to increase survival and mitigate late effects. The zeitgeist in the field of cancer biology as a whole is that this can be done by utilizing molecular biology to discover and create new therapies that target the cancer cells specifically (Baudino 2015). This is done in the hope that cancer cells will be targeted and other developing tissues will

be spared. Pediatric cancers also have the unique condition that many diseases are though to arise from cells of developmental origin and therefore may have special characteristics and vulnerabilities not present in adult cancer (Federico, Brennan, and Dyer 2011).

Germ Cell Tumors

Germ cell tumors (GCT) are a diverse set of malignancies that present in a bimodal distribution with one peak, pediatric germ cell tumors (pGCT), around the time of birth and early childhood and a second peak, adult germ cell tumors (aGCT), around the onset of puberty through early adulthood (Frazier and Amatruda 2009). This disease represents about 3% of tumors in children and the most common cancer of young men (Poynter, Amatruda, and Ross 2010). Though, like many other cancers, the survival rate of patients diagnosed with GCTs has been increasing over the last decade, the incidence in many populations is on the rise for unknown reasons. Thus, study into the underlying developmental and oncogenic mechanisms of this set of diseases is warranted.

The germ in GCT does not allude to the histological appearance of the malignancy rather it refers to the likely cell of origin, a germ cell in early stages of development. This was discovered through an interesting set of observations which linked etiological phenomena to characteristics of the developing germ line (Oosterhuis and Looijenga 2005). The relationship between germline development and GCT risk is discussed at length in the following chapter.

Germ cell tumors are histologically diverse tumors (Figure 1). GCTs present in two different categories based on the precursor lesion and on the histology of the tumor, and both groupings have diagnostic significance. The first distinction is the classification of Type I and

Type II germ cell tumors. This distinction, while generally sorted by age, with Type I in children and Type II in adults, is based on the association with a presumptive precursor lesion known as germ cell neoplasia in situ (GCNIS) (Berney et al. 2016). GCNIS is a histologically diagnosed cell pattern usually seen in the normal tissue adjacent to the tumor (Skakkebaek 1978). It is comprised of undifferentiated germ cells that have proliferated within a seminiferous tubule (Rajpert-De Meyts 2006). It is thought that this lesion correlates to the neoplasia seen in other types of adult tumors prior to full tumor initiation. Type I tumors lack this lesion and are thought to develop from germ cells at an earlier stage. This developmental origin may account for the observed genetic and histological differences between adult and pediatric GCTs. Secondarily germ cell tumors are classified into two groups based on histology. These groups are seminomatous GCTs (SGCT) and non-seminomatous GCTs (NSGCT). The seminomatous GCTs are tumors which are made up of undifferentiated germ cells which can histologically resemble early spermatogonia, oogonia, or even germ cells from developmental lineages. These tumors are called seminoma when present in the testis, dysgerminoma when present in the ovary, and germinoma when found in an extragonadal site. These tumors are generally more susceptible to radiation and chemotherapy and have a better outcome overall. The non-seminomatous GCTs are a group of tumors with many histologies. The tumors from this group may present as a pure form with only one histology or as an amalgam of multiple types which can also include foci of seminoma. This formation is known as mixed malignant germ cell tumor (MMGCT). The primary histologies of NSGCT are embryonal carcinoma (EC), yolk sac tumor (YST), teratoma (TER) and choriocarcinoma (CC). EC is comprised of undifferentiated cells that histologically resemble embryonic cells from the blastocyst. These tumors generally present as Type II GCT.

The cells have been confirmed as pluripotent in culture and can differentiate to any of the three somatic germ layers. YST is the most common malignant pGCT. These tumors have a very complex endodermal morphology with components of both embryonic and extraembryonic endoderm. YSTs can be detected in through analysis of alpha fetoprotein (AFP) in the serum. YSTs are present in both Type I and II GCTs. TER are generally benign tumors in pediatric patients and can also be found as components of MMGCTs. TER histologically presents as a disordered mixture of differentiated cell types from all three somatic germ layers. Infrequently a component of a TER may acquire a unique neural differentiation state. This is known as an immature teratoma and must be treated differently. The CC is a very rare histology and represents trophoblastic differentiation. In general, NSGCTs have worse response to standard therapies and are more resistant to traditional therapies.

The Molecular Genetics of Pediatric Germ Cell Tumors

Most of the genetic aberrations known are related to copy number variation (CNV) of large chromosomal regions, and aneuploidy is a common phenomenon. The most frequent lesions seen in Type II patients are amplification of 12p, usually through creation of isochromosome 12p, and amplification of the X chromosome. However, X amplification is an uncommon event in pediatric germ cell tumors and 12p gain, while present, is infrequent (Palmer et al. 2007). Instead, the pure YST, the most common malignant Type I GCT, has been shown to most commonly possess gains of 1q, 11q, 20q and 22 and loss of 1p, 6q, and 16q (Summersgill et al. 1998). Germ cell tumors also exhibit loss of imprinting which is a normal cancer associated phenomenon. This loss of imprinting is partial in Type I and more complete in Type II GCTs

which may also be related to the erasure of imprinting that takes place naturally in the cell of origin for GCTs and suggests different stages of germ cell development as cells of origin (Ross et al. 1999).

Little is known about the mutational status of pGCTs. However, in GCTs from older patients a study by Litchfield et al. revealed multiple recurrent mutations in KIT, PKPRIR, CDC27, H2AFV, and others (Litchfield et al. 2015). This evidence further supported the notion that KIT mutations are a driver in adult GCTs through activation of the MAPK pathway (Kemmer et al. 2004). Somatic mutations in *BRAF*, another MAP kinase pathway member, have been associated with cisplatin resistance in adult TGCTs (Honecker et al. 2009). Furthermore, exome sequencing of adult GCTs identified somatic mutations in genes including *CIITA, PDGFRA*, *NEB, WHSC1* and *SUPT6H (Brabrand et al. 2015).* In pediatric GCTs very little is known about recurrent mutations. Addeo et al. found that BAX mutations in pGCTs correlated with outcome. There is also some evidence suggesting that p53 mutations may play a role in pGCTs as they do in many other cancer types (Addeo et al. 2007).

Another finding within GCTs is that the landscape of small noncoding RNAs is changed relative to normal gonad. Murray at al. found that total miRNA correlated with the SGCT/NSGCT distinction, with NSGCT expressing higher levels of miRNAs which they suggest correlates with the differentiated state in those tumors. This was driven by the observation that miR372 and miR373 allowed proliferation in RAS active p53 wild-type cells (Voorhoeve et al. 2006). These miRNA were expressed in GCTs from patients as confirmed by miRNA qRT-PCR (Gillis et al. 2007). Furthermore, expression of miR-371~373 and miR-302 clusters was able to segregate malignant GCTs from benign samples (Palmer et al. 2010). The

miR-371~373 cluster was later independently associated with relapsed tumors that had acquired cisplatin resistance (Port et al. 2011). These and subsequent studies suggest a possible role for miRNA profiling in GCT diagnosis and risk stratification.

Hepatocyte Nuclear Factor 4 Alpha

Hepatocyte Nuclear Factor 4 Alpha (HNF4A) is an endodermal transcription factor responsible for differentiation and maintenance of endodermal tissues. HNF4A is a member of the nuclear hormone receptor family of transcription factors (Sladek et al. 1990). HNF4A resides in the nucleus and forms a homodimer which binds DNA in a sequence specific manner and controls transcriptional activity of bound genes. HNF4A is comprised of a variable N-terminal AF-1 domain, a conserved DNA binding domain (DBD), a conserved hydrophobic ligand binding domain (LBD), an AF-2 domain and an F domain (Figure 1). The AF domains are responsible for interaction with many other transcriptional co-regulators. The F domain is an autoregulatory domain at least partially responsible for repression of AF-2 function. A transcriptional variant of HNF4A possess 10 additional amino acids between the AF-2 and F domains and mitigates their interactions (Sladek et al. 1999; Hadzopoulou-Cladaras et al. 1997). The ligand of HNF4A is linoleic acid which binds reversibly to the LBD (Yuan et al. 2009). The function of linoleic acid in this role is still not fully understood. The LBD may serve as a site for novel small molecule based HNF4A modulatory drugs. HNF4A is responsible for regulation of genes responsible for endoderm differentiation and function (Tanaka et al. 2006). HNF4A is expressed in most endoderm derived tissues in both the adult and during development (Kyrmizi et al. 2006; Wang et al. 2000; San Roman et al. 2015). A great deal of interest is placed on this

gene due to its role in a form of type I diabetes known as maturity-onset diabetes of the young (MODY) in which mutations in the HNF4A gene or promoter result in a diabetic phenotype (Furuta et al. 1997; Gragnoli et al. 1997; Stanik et al. 2014). In subsequent years this important transcription factor has been implicated in the development of many other diseases, notably cancer.

In addition to its numerous functions and tissues of expression, HNF4A is a gene with complex structure and multiple isoforms (Figure 2). These isoforms are spatially and temporally regulated primarily through the existence of two independent promoters. These two promoters, known as P1 and P2, are distinct with the proximal P1 promoter nearer the common exons and the distal P2 promoter ~40kb upstream (Taraviras et al. 1994; Furuta et al. 1997; Thomas et al. 2001). Each promoter possesses a distinct first exon and then shares the remaining 11 exons. The P1 first exon encodes part of the AF-1 domain and the P2 first exon does not. Therefore, the P2 has no AF-1 function and can participate in fewer protein-protein interactions. There are other splicing events that can take place in the C-terminal domains of HNF4A which can alter transcriptional activity. In all each promoter based isoform family can code for at least 6 transcripts though some transcripts may not normally be transcribed (Huang, Levitsky, and Rhoads 2009). P1 transcripts include HNF4a1-6 and P2 transcripts include HNF4a7-12. The functional differences between transcripts from the different promoters have been illustrated to be based on both direct DNA binding of transcriptional repressors or coactivators as well as through isoform specific post-translational modification (Eeckhoute et al. 2003). One example is that Src can phosphorylate, and inactivate, HNF4A through targeting of the AF-1 domain which

is only present in the P1 variants of HNF4A (Chellappa et al. 2012; Torres-Padilla, Fougere-Deschatrette, and Weiss 2001).

HNF4A has been implicated in many different cancers though its role is still ambiguous and it lacks the traditional characteristics of a tumor suppressor or oncogene likely owing to its variable functions across different tissues. A general caveat to the discussion of HNF4A's role in disease is that many studies fail to differentiated between different promoter derived isoforms. In the context of the liver, HNF4A is thought to act as a tumor suppressor in that mouse models of HNF4A addition and removal have pointed to HNF4A as a driver of differentiation in this tissue. Loss of HNF4A leads to development of hepatocellular carcinoma (HCC) while exogenous induction of HNF4A in dedifferentiated hepatoma cells results in hepatic differentiation. However, an important paper by Tanaka et al. revealed that some tumors possess isoform specific expression of HNF4A in which P2 derived HNF4A remains expressed in cancer which native P1 HNF4A variants are lost.

Another model of endodermally derived cancer in which HNF4A may play a role is colorectal cancer (CRC). In CRC HNF4A isoforms are variously present between different histologies. In this cancer HNF4A CNV gains have been found which may account for its dysregulation. In normal colon tissue HNF4A is spatially regulated with P1 present in the differentiated compartment and the transit amplifying (TA) compartment of the crypt while P2 proteins are present in the TA compartment and the crypt base stem cell niche. Through an exon swapping model the Sladek lab revealed that colon tissues possessing only P1 or P2 derived HNF4A isoforms react differently to injury and that the P2 variant seemed to predispose mice to colitis and colon cancer while P1 was protective against these lesions (Chellappa et al. 2016).

Furthermore, HNF4A isoform specific induction in a CRC cell line conferred differential oncogenic potential in a xenograft model. In this case P2 increased invasiveness without increasing growth based tumorigenic potential and P1 seemed again to serve a tumor suppressive function. This result is suggested to be based on the interaction between HNF4A and the TCF transcription factors which work closely with the WNT pathway (Vuong et al. 2015). The authors suggest that various promoter derived HNF4A isoforms have complex relationships with WNT signaling which can be either inhibitor or cooperative depending on the context due to competition with CTNNB1 for promoter occupancy.

Besides its role in tumors of endodermal origin, HNF4A has been implicated in other tumor types. In these settings, HNF4A seems to have context dependent roles in cancer. In Neuroblastoma HNF4A acts in an oncogenic role. In this tumor HNF4A is oncogenic with expression linked to increased invasiveness, metastasis, and angiogenesis. This function is mediated by its direct transcriptional target MMP-14. HNF4A is present in the mucinous subtype of ovarian cancer, though its role is unknown. Subsequently, Guo et al. suggested that HNF4A may confer the mucinous phenotype in invasive mucinous adenocarcinoma of the lung (IMA). Furthermore, they found that this HNF4A signature correlates to mucinous cancer variants in gastrointestinal, pancreatic, and breast cancer. This may suggest a role for HNF4A that is divergent from its tumor suppressive role in HCC and CRC. This differential role may involve HNF4A conferring an endodermal fate of one tissue on the developing tumor of another tissue. This transdifferentiation would then provide selective advantage to the clonal population of cells and increase the oncogenic potential of the tumor. This observation opens a new avenue into HNF4A cancer research in which HNF4A acts as an oncogene by differentiating non-

endodermal cancer precursors to rapidly dividing endoderm. This finding may be related to the fact that many new cancer cases develop from tissues of endodermal origin such as lung, prostate and colorectal cancer. Perhaps there are genetic factors that predispose endodermal tissue to oncogenesis which can be coopted by non-endodermal tissues through activation of HNF4A or other endodermal TFs.

HNF4A in Endoderm Development

HNF4A is an important regulator of endoderm development at all stages of development. Knockout of HNF4A in mice leads to failure of visceral endoderm formation and lethality (Li, Ning, and Duncan 2000). In fact, HNF4A is expressed in the primitive endoderm shortly after implantation and is expressed in endodermal derivatives throughout embryogenesis within both embryonic and extraembryonic endoderm (Duncan et al. 1994). Much later it was shown that both P1 and P2 isoforms of HNF4A are expressed in the primitive endoderm with P1 isoforms appearing first (Vuong 2014). Little more is known about the isoform specific expression of HNF4A during embryonic development with the exception of the fetal liver in which primarily P2 promoter variants are found. In general, HNF4A P1 tends to have higher transactivation potentials on genes related to adult development while HNF4A P2 tends to have more potent transactivation potential for genes expressed during fetal development (Torres-Padilla, Fougere-Deschatrette, and Weiss 2001). This dichotomy seems to further confirm the divergent functions of different HNF4A isoforms.

HNF4A has been utilized in different differentiation assays based on embryonic stem cells (ESC) or induced pluripotent stem (iPS) cells. HNF4A overexpression in mouse ESCs was

insufficient to induce endoderm differentiation (Liu et al. 2013), though another later study showed that HNF4A was sufficient to induce primitive endoderm gene expression in a similar system (Yahoo et al. 2016). Multiple endodermal differentiation protocols utilize HNF4A in a sequential fashion to differentiate ESCs to hepatocytes or other endodermal tissue (Takayama et al. 2012; Yamamizu et al. 2013). This includes human umbilical mesenchymal stem cells (huMSC) which are a new area of interest in regenerative medicine. In this system HNF4A was sufficient to induce "highly functional hepatocytes" (Hang et al. 2014). HNF4A and another endodermal TF FOXA2 have been called pan-endodermal TFs and studies have revealed that these two genes in conjunction with other factors, such as GATA4, GATA6, or HHEX, are responsible for context specific expression of endoderm genes (Alder et al. 2014).

One notable interactor of HNF4A in the context of both normal development and cancer is the WNT pathway. The WNT pathway is a signal transduction mechanism that is responsible for functions related to differentiation and stem cell control (Nusse 2008). This pathway is activated when WNT ligand proteins bind to members of the Frizzed (Fzd) transmembrane receptor family (Bhanot et al. 1996). This interaction can be modulated through various proteins which can inhibit or enhance ligand binding or receptor activation such as LRP5/6 or SFRP (He et al. 2004; Zhong et al. 2007). After ligand binding to Frizzled receptors they recruit Disheveled family of proteins (Chen et al. 2003). These proteins interact with Axin, a scaffolding protein, to destabilize its interactions (Mao et al. 2001). Axin is responsible for holding together a group of proteins known as the destruction complex which is comprised of Axin, Glycogen synthase kinase 3 beta (GSK3B), and Adenomatous polyposis coli (APC) which are responsible for destruction of the key mediator of WNT pathway activation, Beta-Catenin (CTNNB1) through

phosphorylation by casein kinase I (Pronobis, Rusan, and Peifer 2015; Yanagawa et al. 2002; Amit et al. 2002). Most CTNNB1 in cells is either sequestered to the membrane in conjunction with junctional complexes and E-cadherin or is destroyed by the destruction complex. Upon destabilization of the destruction complex CTNNB1 is freed to enter the nucleus and drive transcriptional control (Salic et al. 2000). CTNNB1 is known to interact with multiple TFs with the primary interactors being the TCF/LEF transcription factors (Brannon et al. 1997). Figure 3 provides a basic schematic of WNT pathway activation. In normal development regulation of the WNT pathway controls differentiation of many tissues. In the context of cancer, the WNT pathway is generally activated to drive proliferation. This activation is frequently driven by activing mutations in CTNNB1 that prevent interaction with the destruction complex or mutations in destruction complex members that prevents CTNNB1 destruction (Chan et al. 2002).

With regard to HNF4A, WNT has been revealed to interact through HNF4-TCF physical interactions which likely serve as cell specific controllers of gene regulation (Gougelet et al. 2014). In multiple endodermal tissues, it has been shown that HNF4A interacts with the WNT pathway to control proper tissue differentiation and homeostasis. HNF4A interacts with WNT to control liver zonation by competing for promoters involved in epithelial-to-mesenchymal transition (EMT) (Yang et al. 2013). In the intestinal epithelium HNF4A competes with CTNNB1/TCF4 and this also provides a spatial differentiation program to separate proliferative and differentiated compartments.

Summary

Pediatric cancers represent a large set of relatively rare diseases the treatment of which are relatively non-specific and which are generally underrepresented in basic biomedical science research. The focus in the field is to develop more specific molecular therapies to improve survival and abrogate late effects. Germ cell tumors are a subset of pediatric tumors which are derived from primordial germ cells. These tumors affect both children and adults and are the most common cancer of young men. These tumors can present with a wide array of germ-line, somatic, or extraembryonic histologies and little is known about their etiology. A specific histological subtype of GCTs, the yolk sac tumor, is an endodermal tumor that is the most common GCT in young children. This tumor is formed by endodermal differentiation from the pluripotent PGC.

Hepatocyte Nuclear Factor 4a is a nuclear hormone receptor that is involved in endoderm development, differentiation, and maintenance. This gene was originally discovered for its role in diabetic phenotypes but it has since been revealed to play an important role in many diseases, specifically cancer. This gene has been shown to be a powerful tool in endoderm specific differentiation in a number of systems. Its role seems to be two-fold in cancer. In the context of some endodermal tissues it interacts with the WNT pathway in an isoform specific fashion and is involved in tissue homeostasis, the interruption of which can lead to neoplastic transformation under certain conditions. In the context of tissues which lack HNF4A it may confer an endodermal phenotype that promotes oncogenicity. The study of this gene and its isoform specific effects in cancer may be an important step in understanding the molecular mechanisms controlling tumors of endodermal histology such as YSTs.

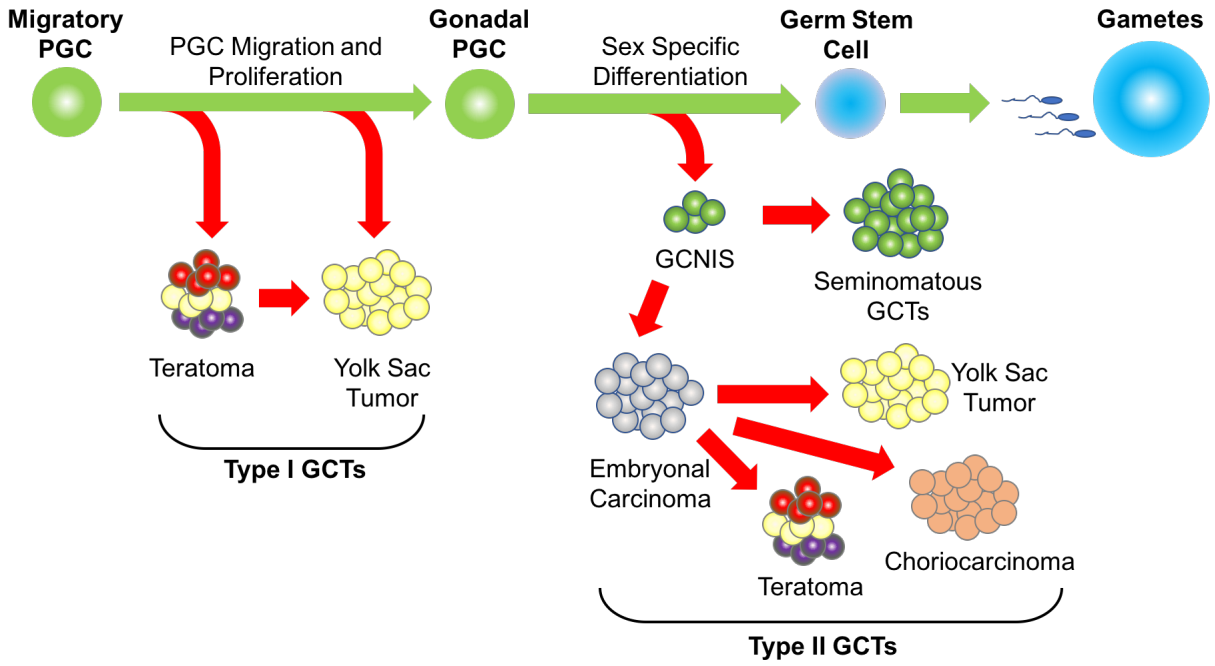

Figure 1.1. GCT Development and Histologies.

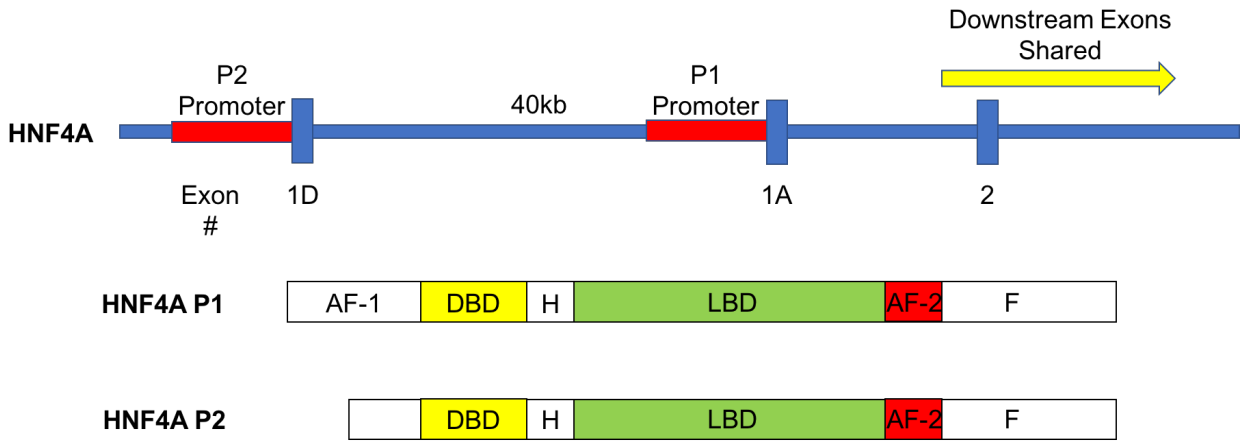

Figure 1.2. HNF4A Gene and Transcript Structure.

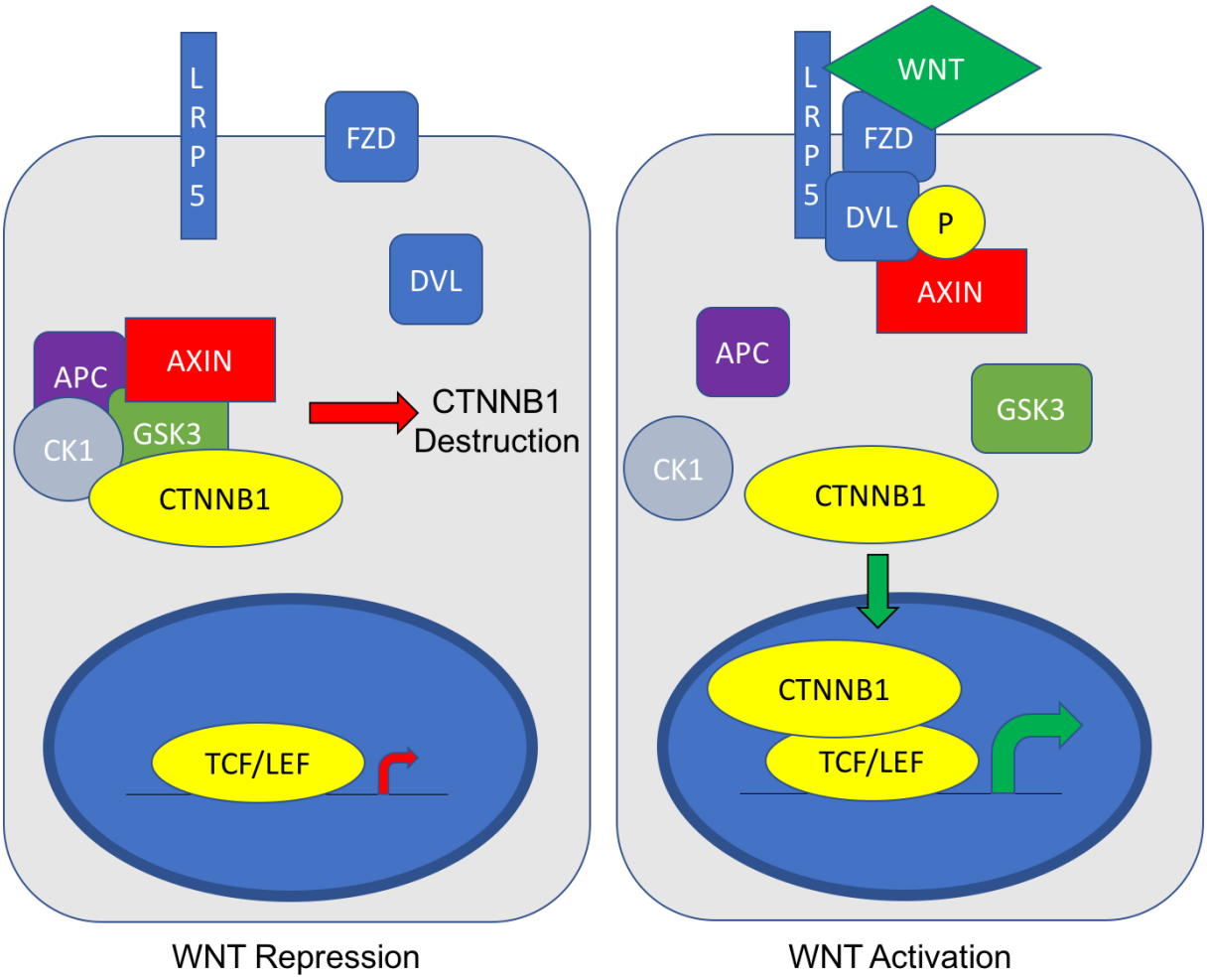

Figure 1.3. The WNT Signaling Pathway.

Chapter II

Hepatocyte Nuclear Factor 4 alpha is Responsible for Isoform Specific Regulation of Yolk Sac Tumor Differentiation and Maintenance

Abstract-

Yolk sac tumors are a histological category of germ cell tumors, which represent the most frequent malignancy in young men. Little is known about the molecular mechanisms responsible for the aberrant differentiation and oncogenic potential of this disease. Multiple recurrent chromosomal copy number variations are the primary genetic lesions discovered in this disease. Frequent gains of chromosome 20q13 provide clues as to the identity of important YST driver genes. We have determined that the Hepatocyte Nuclear Factor 4 alpha locus, which resides in this region, is frequently amplified in YSTs. Overexpression of HNF4A in an undifferentiated GCT line is sufficient to drive endodermal gene programs. Additionally, isoform specific manipulation of HNF4A revealed differential oncogenic potential amongst HNF4A isoforms. This isoform specific manipulation was involved in modulation of the WNT pathway which has previously been identified as active in YSTs. These results allowed us to uncover HNF4A isoform specific differences in histological samples from YST patients. Our findings reveal a possible new druggable target in YST treatment and reveal an interesting isoform dependent mechanism for understanding tumor development.

Introduction-

Germ cell tumors (GCTs) are malignant neoplasms derived from the primordial germ cell (PGC) that occur in neonates, children, adolescents and young adults. GCTs make up about 3% of all cancers in neonates and young children and about 15% of cancers in adolescents. Testicular germ cell tumor (TGCT) is the most common cancer in men between the ages of 15 and 40 (Frazier and Amatruda 2009; Poynter, Amatruda, and Ross 2010). GCTs exhibit multiple histological forms with differing gene expression profiles and cellular morphologies (Oosterhuis and Looijenga 2005). Broadly speaking these tumors fall into two classifications with regard to histology and treatment. The first group, seminomatous GCTs, histologically and genetically resemble undifferentiated germ cells akin to PGCs or germ line stem cells as evidenced by expression of germ cell markers such as *NANOG* and *KIT* (Frazier and Amatruda 2009). The second group, non-seminomatous GCTs, exhibit a much wider variety of histological appearances that resemble each of the embryonic and extra-embryonic germ layers as well as the pluripotent embryonic stem cells themselves. Embryonal carcinoma (EC) is an undifferentiated tumor that expresses multiple pluripotency markers including *POU5F1* and resembles embryonic stem cells (Przyborski et al. 2004). Teratomas exhibit somatic differentiation into derivatives of all three germ layers, and are usually benign with rare cases of immature teratomas proving malignant. Yolk sac tumors (YSTs), which were named for their histological similarity to rodent yolk sac, are a diverse class of GCTs that present with extremely varied histologic appearances generally resembling some form of endodermal tissue. Neonates and young children aged less than 5 years present most frequently with teratomas and YSTs while disease in adolescents and young adults can present as a pure tumor of any histology or as a mixed malignant germ cell

tumor (MMGCT), comprised of multiple different histological subtypes. Both classifications of GCTs respond well to cisplatin based chemotherapeutic regimens, however these treatments frequently result in severe secondary effects such as infertility, hearing loss, and risk of second malignancies (Frazier and Amatruda 2009). More specific, targeted therapies for GCTs have yet to be developed.

Recent data have begun to reveal something of the molecular mechanisms of GCT development. Gain of chromosome 12p has long been known to be pathognomonic of TGCT (Atkin and Baker 1982). In young children, the most common GCT chromosomal imbalances are gains of chromosomes 1p and 20q and the loss of chromosome 1p and 6q (Palmer et al. 2008). Genome-wide association studies have identified a number of loci associated with risk of developing GCT, including *KITLG*, *SPRY4*, and others, however the functional significance of these links remains unknown (Pyle and Nathanson 2016; Palmer et al. 2008). Yolk sac tumors are particularly interesting due to their occurrence in both a pure form in neonates, young children and in the ovary of adolescents and young women, and as a component of MMGCT in adolescents and adults. Based on murine experiments in which implanted undifferentiated cells resulted in a tumor histologically resembling endoderm it is thought that YSTs are an endodermal differentiation from ECs when found as part of an MMGCT (Sobis, van Hove, and Vandeputte 1982; Pierce and Dixon 1959; Pierce et al. 1962). Additionally, human iPSC teratoma assays that show microscopic YST foci (Unzu et al. 2016). Currently these tumors are distinguished from other GCTs from their expression of *GPC3* and *AFP* and can frequently be detected through serum AFP measurements (Zynger et al. 2010). YSTs express markers of

embryonic endoderm, such as *GATA4* and *GATA6*, as well as CTNNB1 staining which is frequent in normal endodermal tissues (Mannisto et al. 2005; Fritsch et al. 2006).

Hepatocyte nuclear factor 4 alpha (HNF4α, encoded by the *HNF4A* gene) is a nuclear hormone receptor transcription factor that resides at the 20q13 locus (Sladek et al. 1990). HNF4α is an endoderm specific transcription factor responsible for the expression of many important endoderm specific gene products in multiple different endodermally derived tissues (Zhong, Sladek, and Darnell 1993; Sladek 1994; Ihara et al. 2005). It was originally identified through its role in transcriptional control of liver specific genes in rats, and has since been implicated in the development and maintenance of endoderm throughout all stages of development REF. Loss-of-function mutations and promoter polymorphisms of *HNF4A* have been linked to Type I Maturity Onset Diabetes of the Young (MODY1) (Yamagata et al. 1996). *HNF4A* is transcribed from two distinct start sites controlled through distinct promoter regions. The distal P2 promoter lies approximately 40kb upstream of the proximal P1 promoter and is involved in both fetal endoderm development and adult homeostasis while the P1 promoter is primarily expressed in adult tissues (Nakhei et al. 1998; Torres-Padilla, Fougere-Deschatrette, and Weiss 2001). Both of these promoters are responsible for at least three transcripts with different C-terminal configurations, however the primary difference between the two isoforms is the N-terminal AF-1 domain which is present in only P1 derived transcripts. This structural difference was shown to be functional through exon swapping experiments in murine livers which resulted in transcriptional changes (Briancon and Weiss 2006). Furthermore, through this work and others, HNF4α isoforms have been shown to co-express in many endodermal tissues

with cell type specific expression (Chellappa et al. 2016; Li et al. 2006). HNF4α isoforms have been further characterized by differences in DNA binding specificity, cofactor interaction, and post-translational modification which all have P1/P2 specificities (Bogan et al. 2000; Chellappa et al. 2012). Over the last decade HNF4α has become the focus of multiple studies relating to the development of cancers in both endodermal and non-endodermal tissues. These studies have suggested that HNF4α may act as an oncogene or tumor suppressor in both an organ-specific as well as isoform-specific fashion. For example, in liver the predominantly expressed P1 isoform is responsible for maintaining the differentiated state and protecting against development of hepatocellular carcinoma (Yin et al. 2008; Hatziapostolou et al. 2011; Santangelo et al. 2011). In some tissues which lack endogenous *HNF4A* expression, HNF4α P1 and P2 are aberrantly expressed in tumors of a mucinous phenotype suggesting oncogenic action through inappropriate differentiation (Sugai et al. 2008; Sugano et al. 2013). Strikingly, functional studies have shown that *HNF4A* expression in neuroblastoma promotes invasion and metastasis through regulation of MMP14 (Xiang et al. 2015). In colorectal cancer, *HNF4A* amplifications are a frequent event that correlates with subtype (Zhang et al. 2015). In a CRC xenograft model of doxycycline inducible HNF4α the P1 isoforms suppressed tumor growth while P2 isoforms did not (Vuong et al. 2015). Moreover, removal of only P1 Isoforms of HNF4α, in an *in vivo* model of CRC, have revealed that P2 expression alone cannot perform HNF4α's tumor suppressive functions in this context (Oshima et al. 2007; Chellappa et al. 2016). In summary, many tumor types exhibit aberrant regulation of *HNF4A* isoforms (Tanaka et al. 2006). These results suggest that HNF4α isoforms are important in cancer development and that their functional roles should be determined on a case by case basis to develop more specific targeted therapies.

Here we show that aberrant expression of *HNF4A*, frequently driven by focal amplification events, plays a role in YST differentiation and maintenance. HNF4α is sufficient to drive primitive endoderm gene expression in a GCT in vitro model. HNF4α is robustly expressed in YSTs and exhibits isoform specific cell localization. This localization is related to expression of CTNNB1 which suggests a functional role for HNF4α in YST maintenance. *In vitro* isoform specific knockdown of *HNF4A* reveals HNF4A P1 transcripts as responsible for maintenance of growth. These results indicate both a new mechanism for the development of YSTs and suggest new targets for development of therapeutics.

Methods-

Immunoblotting

Total cell lysates were prepared by lysing the cells with RIPA sample loading buffer containing protease inhibitor mixture (Roche) and 2.5% (vol/vol) beta-mercaptoethanol (Sigma). Immunoblotting was conducted with antibodies against HNF4A (H1415 Invitrogen; 1:1,000), FLAG (Cell Signaling; 1:5000), and Actin (Sigma; 1:5,000). Goat anti-mouse HRP Conjugated secondary antibody (Bio-Rad; 1:10,000) or Goat anti-rabbit HRP Conjugated secondary antibody (Bio-Rad; 1:10,000) were used and protein was detected using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher).

Cell Culture

GCT44 and NTERA2 cells were grown were grown in DMEM (Sigma) supplemented with 10% (vol/vol) FBS (Sigma) and 1411H cells were grown were grown in DMEM without sodium pyruvate (Sigma) supplemented with 10% high quality FBS (Thermo Fisher), in a 37 °C humidified incubator containing 5% CO_2. Media was replaced every two days and cells were split 1:5 using either .05% Trypsin EDTA (Invitrogen) for GCT44 and NTERA2 or .25% Trypsin EDTA (Thermo Fisher) for 1411H.

Crystal Violet Assay

Cells were seeded at 35,000 cells/well in cell culture treated 12 well plates (COSTAR). 24 hours after plating cells were treated with siRNA against P1 *HNF4A* or P2 *HNF4A* or a control siRNA (Thermo Fisher) as described previously (Chellappa et al. 2016) for 24 hours using Lipofectamine RNAimax transfection reagent (Thermo Fisher). Plates were then collected at days 0, 3, 6, 9, and 12 and fixed in formalin. Plates were stained with crystal violet and solubilized in 10% glacial acetic acid then staining was quantified at 590nm (POLARstar Omega, BMG Labtech).

AFP ELISA

ELISA was performed using Quantikine ELISA kit targeting Human α-Fetoprotein using standard protocol for cell culture media. Media from human YST line GCT44 was collected after 2 days of culture as a positive control. NTERA2 cells stably transduced with inducible *HNF4A* were treated with Doxycycline for 7 days with media collection at 48 hours and 7 days with or

without *HNF4A* induction. At both time points media collected was exposed to cells for 48 hours prior to collection.

qRT-PCR

Cells were collected by trypsinization and pelleted. Cells were processed with QIAshredder (Qiagen) and RNA was processed using the RNeasy kit (Qiagen). cDNA was prepared using the RT2 HT First Strand Kit (Qiagen). qRT-PCR was performed using iTaq Universal SYBR Green Supermix and run on iScience 7900HT Fast Real-Time PCR System (Applied Biosystems). *HNF4A* primers were purchased from SA Biosciences (PPH05540A-200) and WNT3a primers were purchased from Qiagen (PPH02431A-200). *HNF4A* P1 specific, *HNF4A* P2 specific (Harries et al., 2008), *AFP*, *GATA4*, *GATA6*, *NANOG* (Valera et al., 2008), APOA1 (Kostopoulou et al. 2015), *WNT5a* (Okamoto et al. 2014), *GAD1*, *APCDD1*, *FZD5* (DeAlmeida et al. 2007), and *SALL4* (Zhang et al. 2015) primers are previously described. The remaining primer sequences are as follows CER1 (F- GGATGGCCGCCAGAATCAG; R- TGGCACTGCGACAAACAGAT), LEFTY2 (F- TGGACCTCAGGGACTATGGAG; R- CCGAGGCGATACACTGTCG), WLS (F- TCCCTGGCTTACCGTGATGA; R- GCATTTGAGTTTCCGTGGTACT), and DVL2 (F- GAGGAAGAGACTCCCTACCTG; R- CGGGCGTTGTCATCTGAAAT).

Super TOPFlash Assay

M50 Super 8x TOPFlash was a gift from Randall Moon (Addgene plasmid # 12456). 1411H and GCT44 cells were plated in 6 well plates (COSTAR) and treated with *HNF4A* siRNA as described above. 24 hours after siRNA transfection cells were transfected with the Super

TOPFlash plasmid using the Lipofectamine LTX and Plus reagent. Cells were then incubated for 48 hours. Cells were then collected and firefly luciferase levels detected using Dual-Luciferase Reporter Assay System (Promega) and Luminescence detected on a POLARstar Omega plate reader (BMG Labtech).

Immunohistochemistry

Human tumor microarray possessing yolk sac tumor and seminoma samples were generated at Children's Medical Center Dallas. Samples were stained with isoform specific antibodies against HNF4α P1 (K9218, Abcam) and P2 (H6939, R&D Systems) and counterstained with hematoxylin following the procedure previously established (Tanaka et al. 2006).

Copy Number Analysis

For SNP arrays, genomic DNA was prepared from FFPE sections of clinically-annotated human GCTs, and the DNA was quantified with PicoGreen (Invitrogen, Carlsbad, CA) prior to submitting for OncoScan FFPE Express V2.0 (Affymetrix, Santa Clara, CA). OncoScan is a genome-wide SNP assay based on Molecular Inversion Probe technology (Wang et al. 2007). Copy number data were analyzed with Nexus Copy Number software (Discovery Edition 5.1, Biodiscovery, El Segundo, CA) using human genome assembly GRCh37. Data were analyzed using SNPRank Segmentation, an extension of the Rank Segmentation algorithm where B-Allele Frequency values are also included in the segmentation process generating both copy number and allelic event calls. A minimum of 5 probes with significance threshold set to 5×10^{7} was

required to define a segment. The threshold was set to +/- 0.45 for copy-number gains and losses and +/-1.2 for high level gains and losses, respectively.

RNA Sequencing

For RNA sequencing, adapter removal and quality-filtering was conducted by Cutadapt (1). Alignment to the reference human genome, build hg19, was performed by Bowtie2, which is ultrafast and memory-efficient tool for aligning sequencing reads to long reference sequences. TopHat2 was also be used for alignment. De novo assembly of reads into transcripts and differential expression analysis was performed by Cufflinks and Cuffdiff. Isoforms were defined based on Gencode version 19 (https://www.gencodegenes.org/releases/19.html). Isoform specific expression data was generated by Cufflinks and custom PERL scripts.

Results

***HNF4A* amplifications are a common event in Yolk Sac Tumors and HNF4α exhibits strong expression specific to YSTs.**

Though it has long been known that the chromosomal region 20q13 is frequently amplified in Yolk Sac Tumors (YSTs), the gene or genes providing the selective advantage needed for retention of this amplification is currently unknown. Therefore, this region, which is commonly amplified in YSTs, represents an important opportunity to discover YST driver genes. To determine pattern of genomic copy number variation across a broad range of GCT subtypes, 93 samples with varying histology were subject to SNP analysis. As expected, Yolk Sac Tumors possessed frequent 20q13 amplifications. Interestingly, within the 20q13 interval, the *HNF4A* gene was frequently and focally amplified with 40% of samples exhibiting *HNF4A* amplification

(Figure 1A). *HNF4A* is an endodermal transcription factor required for the development of early endoderm (Chen et al. 1994), and Yolk Sac Tumors resemble primitive endoderm histologically and by gene expression pattern, raising the interesting possibility that *HNF4A* might have a direct role in the initiation or maintenance of the YST phenotype. To investigate this possibility, we tested human YST RNA for *HNF4A* expression level as measured by QRTPCR. Compared to seminomas, *HNF4A* levels were significantly elevated in YSTs (Figure 1B). To ensure that *HNF4A* was transcriptionally active within YSTs we carried out RNASeq on 23 YSTs and 5 Seminomas and performed Gene Set Enrichment Analysis (Subramanian et al. 2005) using a gene set comprised of consensus *HNF4A* target genes. This analysis revealed that *HNF4A* target genes are significantly enriched in Yolk Sac Tumors and infrequently expressed in Seminomas (Figure 2C). Finally, we performed HNF4a immunohistochemistry on a tumor microarray containing 14 Yolk Sac Tumor Samples and 9 Seminomas collected from pediatric patients. We observed that 14/14 Yolk Sac Tumors exhibit robust HNF4a staining while 0/9 Seminomas exhibited staining (Figure 2D).

HNF4A Expression is sufficient to induce a primitive endoderm gene expression signature in a model of Yolk Sac Tumor trans-differentiation.

The discovery of *HNF4A* amplifications and the presence of an HNF4α-driven gene expression program in YSTs prompted us to ask whether *HNF4A* expression is sufficient to induce endodermal gene expression in the germ cell lineage. NTERA-2 cells are a pluripotent human testicular Embryonal Carcinoma (EC) cell line capable of differentiation along multiple

cell lineages representing the three germ layers (Pal and Ravindran 2006; Andrews et al. 1984). Type II YSTs within Mixed Malignant Germ Cell Tumors (MMGCTs) are thought to derive from differentiation of EC to various endodermal histologies (Gopalan et al. 2009). To determine if overexpression of *HNF4A* was sufficient to drive differentiation to primitive endoderm, we created an NTERA-2 cell line expressing *HNF4A* under the control of a doxycycline inducible promoter. This line exhibits rapid and robust induction of HNF4α tagged with MYC and FLAG tags upon addition of doxycycline to the cell culture media as exhibited by immunoblot against HNF4α and QRTPCR for the *HNF4A* transcript (Figure 2A). After 24 hours of HNF4α induction by doxycycline, expression of the HNF4α target genes *APOA1* and *AFP* was significantly increased compared to control cells (Figure 2B). Sustained *HNF4A* expression (7 days induction) led to increased expression of endoderm regulators *GATA4* and *GATA6* as well as primitive endoderm markers *LEFTY2* and *CER1*, as well as decreased expression of pluripotency related genes *NANOG* and *SALL4* had a significant decrease in expression level (Figure 2C). *GATA4* and *GATA6* are master endodermal transcription factors, and *HNF4A* is itself a target of *GATA6* (Morrisey et al. 1998). Consistent with a functional role of GATA6 in our cell model, we observed expression of the endogenous HNF4α gene products (both P1 and P2 isoforms) upon doxycycline induction (Figure 2D). Taken together, these results indicate that expression of *HNF4A* can induce a general endoderm gene expression program, mediating a shift from pluripotent EC fate to an early endodermal fate.

Secreted Serum AFP is a primary clinical diagnostic marker of YST (Frazier and Amatruda 2009). *HNF4A* induction results in increasing secretion of AFP into the media, and AFP production by doxycycline-induced cells was equivalent to that of the YST cell line GCT44

(Figure 2E). Thus, *HNF4A* overexpression in the germ cell lineage drives an endodermal gene expression program and recapitulates key features of yolk sac tumors.

HNF4A overexpression results in elevation of WNT pathway related genes and HNF4A isoform expression is related to WNT pathway regulation in YSTs.

We next performed microarray studies of control and doxycycline-induced EC cells, and asked what cellular signaling pathways might mediate the HNF4α-driven endodermal program in YSTs. Several lines of evidence suggested WNT signaling as a candidate. Embryonic endoderm relies on WNT signaling for differentiation signals, and WNT is intimately tied to growth and expansion of primitive endoderm (Price et al. 2013; Hwang and Kelly 2012). WNT signaling is active in pediatric YSTs (Fritsch et al. 2006). Consistent with a role of WNT signaling, we found that a large number of WNT pathway members and associated genes had significantly altered expression after *HNF4A* long term induction (Figure 3A.) We used QRTPCR to validate the effect of HNF4α on selected WNT pathway genes, including those involved in WNT ligand secretion (*WLS*, *WNT3*, and *WNT5A*), WNT regulation in NTERA2 cells (*GAD1*, *APCDD1*), and WNT signal transduction (*FZD5, DVL2*) (Figure 3B). Thus, one effect of *HNF4A* expression in the germ cell lineage is transcriptional activation of the WNT pathway.

YSTs exhibit robust expression of isoforms from both the P1 and P2 *HNF4A* promoters and demonstrate isoform specific cell staining.

Induced expression of *HNF4A-P1* in embryonal carcinoma cells led to extensive endodermal differentiation in the cells, including expression of the master regulator *GATA6* and induction of both P1 and P2 isoforms of *HNF4A* (Figure 1D). Recent studies in several cancer types have suggested that the P1 and P2 isoforms may have different functions in tumor development (Takano et al. 2009; Tanaka et al. 2006; Vuong et al. 2015). We therefore sought to probe isoform-specific *HNF4A* expression in YSTs. Using RNAseq and an algorithm designed to detect different isoforms we determined that *HNF4A* isoforms expressed from the P1 promoter are the most highly expressed group with all tumors also possessing moderate levels of P2 derived transcripts (Figure 4A). These results were further corroborated by QRTPCR performed on human YST samples using isoform specific primers. These results demonstrated that YST expression of *HNF4A-P1* is hundreds of times higher compared to seminoma sample while *HNF4A-P2* transcripts are elevated to a significant but lesser extent (Figure 4B). To determine the isoform specific expression *HNF4A* pattern within YSTs, we performed immunohistochemistry on a YST TMA using antibodies able to differentiate between proteins derived from the two *HNF4A* promoters through binding to unique N-terminal regions. From this analysis, we recapitulated the previous RNAseq and QRTPCR result that both the P1 and P2 isoforms are expressed in 14/14 YSTs on the TMA and 0/9 Seminoma. The most striking finding of this experiment was that while HNF4α P2 proteins were detected at various levels within nearly all cells within all YSTs there were specific clusters of cells that did not express HNF4α P1 (Figure 4C). These cells tended to have a distinct cell morphology that was

comprised of small, tightly grouped cells with high nuclear to cytoplasmic (N:C) ratio relative to the surrounding P1/P2 positive cells (red dotted areas). Isoform specific differences in HNF4α expression were observed in 4/14 YSTs, though due to their small relative number these rare groupings may have been excluded from other samples on the TMA. These cell type specific differences are further evidence that the *HNF4A* isoform specific expression may be related to functional difference in cell gene expression.

This evidence of WNT activation after *HNF4A* induction led us to investigate possible isoform-specific differences in YST WNT signaling. Previous reports on GCTs have revealed that YST and immature teratomas possess more membranous, nuclear, and cytoplasmic instances of CTNNB1 staining as compared to Seminomas and ECs (Fritsch et al. 2006). CTNNB1, the key regulatory transcription factor of WNT signaling, is a useful protein for determining WNT activation status in cells (Tolwinski and Wieschaus 2004). We therefore performed co-immunofluorescence on YST tissue sections with antibodies specific for CTNNB1, and HNF4α P1 or HNF4α P2. Staining revealed that HNF4α isoform specific expression was closely linked to CTNNB1 expression and localization (Figure 5A). As seen previously the high N:C cells possessed no P1 HNF4A expression (Top) and moderate P2 HNF4A expression (bottom). CTNNB1 exhibited strongest expression in the high N:C P2 positive cells with primarily membranous and cytoplasmic expression. Cells possessing moderate membranous, cytoplasmic and nuclear HNF4A expressed both P1 and P2 isoforms. Finally cells that express little to no CTNNB1 were strongly P1 positive.

Human YST cell lines express *HNF4A* and specific modulation of P1 isoforms results in robust growth inhibition.

The results described above indicate that HNF4α isoforms are expressed in different domains within YSTs, where they may mediate domain-specific effects on WNT signaling and cell proliferation, with important implications for the growth of YSTs *in vivo*. To further probe this hypothesis we performed functional studies in two YST cell lines. The lines GCT44 and 1411H express isoforms from both *HNF4A* promoters at different levels as assayed by both western blot and QRTPCR compared to EC cell line NTERA2 (data not shown). We performed both general and isoform-specific knockdown of HNF4α to determine whether different isoforms could have differential effects on tumor growth. Both GCT44 and 1411H responded to all siRNA treatments with appropriate isoform specific reduction of HNF4α protein levels (Figure 6A). This result was also confirmed by isoform specific QRTPCR which demonstrated appropriate and significant reduction of *HNF4A* isoforms according to the siRNA used (Figure 3B). To ensure that siRNA induced *HNF4A* knockdown was affecting HNF4α transcriptional activity cells were assay for canonical HNF4α target *APOA1* which was significantly reduced in all cases (Figure 6B). Next, we performed isoform specific *HNF4A* knockdown on cells in a growth assay to determine if HNF4α was required for maintenance of an oncogenic rapid growth phenotype. We found that knockdown of the HNF4α P1 isoform resulted in the most severe and significant reduction in cell growth potential in both cell lines (Figure 6C). This result is striking because it is confirmed in both the cell lines despite the fact that P1 is not the most strongly expressed isoform group in the 1411H cells. Thus the presence of the P1 isoform correlates with the relative proliferation of YSTs cells, consistent with the immunofluorescence data on human

tumors (Figure 5B, C). We also noted, however, that P1 *HNF4A* knockdown leaves P2 as the remaining isoform, and that the combination of P1 and P2 specific siRNA, which affects all isoforms of *HNF4A*, had a less robust effect on cell growth in GCT44 cells and had almost no effect in 1411H cells. Therefore, it is possible that the P2 isoforms may possess a growth inhibitory role in the setting of YSTs. To test this possibility, we expressed P2 *HNF4A* under the control of the strong constitutive CMV promoter. Cells were treated with CMV P2 *HNF4A* plasmid and allowed to grow for 7 days with a second transfection on the fourth day of growth to ensure robust expression. Intriguingly cells treated with the CMV P2 plasmid showed a significant growth reduction when compared to either empty vector control (Figure 6D). Taken together, these data indicate that the relative proportion of P1 and P2 isoforms can have significant effects on the rate of growth of YST cell *in vitro* and *in vivo*.

Discussion

This study demonstrates that *HNF4A* is important in maintenance of growth potential in YSTs and may be partially responsible for the endodermal appearance and differentiation of both Type I and II YSTs. This study further highlights the importance of WNT signaling in GCT biology. We demonstrate that *HNF4A* is frequently amplified in YSTs and is a strong candidate for the gene conferring selective advantage to YSTs bearing 20q13 amplifications. We found that nearly half of YSTs possess an amplification of the *HNF4A* locus which is roughly equivalent to previous reports of 20q13 amplification frequency. Subsequently we found that *HNF4A* was expressed in all YSTs as assayed by RNAseq, QRTPCR, and immunohistochemistry. HNF4α is an endoderm specific transcription factor and the histological similarity between YSTs and multiple endodermal tissues provides a straightforward theoretical framework for the possible

role of *HNF4A* in YSTs. This possibility is backed by our evidence that HNF4α transcriptional targets are enriched in YSTs compared to other GCT histologies. Further evidence is the fact that AFP, an important diagnostic marker of YSTs, is a direct transcriptional target of HNF4A (Alder et al. 2014). On the basis of *HNF4A*'s amplification frequency and strong expression in YSTs we hypothesized that *HNF4A* may play an important role in YST development and oncogenesis.

We propose that *HNF4A* amplification may be a mechanism driving the differentiation of YST precursor lesions such as aberrantly migrated PGCs, premalignant CIS, or the EC component of MMGCTs. This would explain both the endodermal characteristics of YSTs as well as the high levels of AFP expression. To determine whether *HNF4A* overexpression is sufficient to drive a large-scale gene expression change towards primitive endoderm we created a cell line that can mimic the acquisition of aberrant *HNF4A* expression in the context of EC. This doxycycline inducible line confirmed that *HNF4A* expression was sufficient to drive multiple levels of endoderm gene expression. *GATA4, GATA6, CER1, LEFTY2, NANOG*, and *SALL4* are not canonical HNF4A targets, yet sustained *HNF4A* expression in an EC context was able to create a genetic environment in which primitive endoderm markers were elevated and pluripotency markers were reduced. Furthermore, HNF4α was able to drive expression and secretion of AFP in this context which further supports the hypothesis that *HNF4A* is responsible for the endodermal character of YSTs. Our hypothesis that *HNF4A* drives a primitive endoderm program was further supported by the fact that long term induction of exogenous *HNF4A*

eventually promoted the expression of endogenous *HNF4A*, a known target of master endodermal transcription factors such as *GATA6.*

With the knowledge that *HNF4A* was sufficient to drive an endodermal signature in a YST precursor we collected two YST cell lines and found that both exhibit robust expression of both isoform families of *HNF4A* further highlighting the prevalence of *HNF4A* expression in YSTs. We used isoform specific siRNAs to determine the possible importance of HNF4A in maintaining the oncogenic potential of YSTs. We found that in both lines the P1 isoform was most strongly linked to the growth potential. This was especially interesting as multiple studies have suggested that in other contexts that the P2 isoforms can be most strongly linked to oncogenic phenotypes (Vuong et al. 2015; Oshima et al. 2007). Furthermore, addition of exogenous P2 HNF4A to both cell lines resulted in significant growth inhibition. Our results may indicate that increase of HNF4A expression in non-endodermal tissues may represent the oncogenic potential of the gene. In these tissues HNF4A drives an endodermal differentiation program which confers selective advantage to tumor cells. This would stand in opposition to HNF4A expressing tissues in which HNF4A is responsible for maintenance of normal endoderm differentiation. This would explain why gain of HNF4A in tissues that express very little HNF4A can be oncogenic while loss of predominant isoforms of HNF4A can result in loss of tumor suppressive function (Xiang et al. 2015). Additionally, the relative amount of each HNF4A isoform in a given tissue may play a role in overall growth control as evidenced by the seemingly synergistic effect on growth in the isoform combination siRNA and CMV *HNF4A* experiments.

This result could rely on the fact that *HNF4A* isoforms exhibit inter-isoform heterodimerization which could result in novel interactions with cofactors.

We further demonstrate the isoform specific differences in YST growth are not limited to the *in vitro* setting. We noted intra-tumoral differences in cell morphology that correlated with *HNF4A* isoform expression. Based on these morphological differences we sought a mechanism by which HNF4A could regulate YST growth and morphology. A microarray of doxycycline induced *HNF4A* revealed multiple direct HNF4A targets as well as a few interesting WNT related genes. Additionally, several studies have suggested that HNF4A and its isoforms can interact with the WNT pathway both cooperatively or antagonistically (Colletti et al. 2009; Vuong et al. 2015). We therefore investigated whether HNF4A expression was linked to WNT pathway activation in the context of GCTs. We then found that HNF4A isoform specific expression was related to the WNT subcellular localization status of YST samples. Furthermore, HNF4A expression levels from patients were correlated with CTNNB1 levels such that the P1 isoforms were more closely correlated with CTNNB1 levels. Finally, we characterized a functional relationship between modulation of HNF4A isoforms and WNT pathway activity. Through multiple cell based assays we determined that HNF4A P1 expression was related to the regulation of WNT activity.

This work provides a new direction for investigation into the molecular underpinnings of YST oncogenesis. Our data provides a feasible model for the development of this endodermal malignancy in which aberrant overexpression of *HNF4A* by gene copy number increase, or some other unknown mechanism, creates an endodermal gene signature that results in WNT pathway

activation and confers a selective advantage (Figure 7). In our model, the pluripotent status of precursor PGCs provides a developmental vulnerability in Type I GCTs such that mis-migration or improper development cues can lead to formation of teratoma that terminally differentiate. However, amplification of the *HNF4A* locus results in trans-differentiation to primitive endoderm which is licensed for growth by its lack of developmental cues. This model is supported by the fact that the primitive endoderm in mice is able to form YSTs after transplantation into a syngeneic subject (Sobis, Verstuyf, and Vandeputte 1993). Furthermore, HNF4A induced primitive endoderm formation may license the neoplasm to grow without terminal differentiation which allows for the acquisition of further oncogenic mutations and could explain the benign/malignant dichotomy between teratoma and YST. In the case of Type II GCTs, in which oncogenic status has already been acquired, *HNF4A* amplification results in endodermal differentiation which may provide selective advantage based on the nutrient processing mechanisms possessed by endodermal tissues though that hypothesis must be investigated further. The *HNF4A*/WNT expression pattern observed by IF recapitulates, an albeit disorder, system that is similar to other endodermal tissues (Figure 7). This further supports the notion that HNF4A acts as a transdifferentiating agent rather than a traditional oncogene.

The WNT active state of YSTs may then serve as a potential target for molecular therapies such as WNT pathway inhibitors which we have previously shown to be efficacious in YSTs. Furthermore, much remains to be explored with regard to other potential isoform specific HNF4A targets that may play a role in YST maintenance. There may be key endoderm specific features genetically controlled by *HNF4A* such as energy metabolism or xenobiotic chemical detoxification that will prove targetable nodes in YSTs. Our hypothesis that *HNF4A* is

responsible for trans-differentiation from PGC to primitive endoderm suggests that Type I YSTs may be susceptible to some form of differentiation therapy which will provide appropriate terminal differentiation cues. Additionally, we have further added to the expanding field of HNF4A isoform biology in regard to cancer. HNF4A targets that exhibit isoform specific regulation may be the key to finding molecular targets for treatment in many endodermal malignancies which possess aberrant regulation of HNF4A isoforms. HNF4A itself may prove to be an interesting oncogenic target through its ligand binding domain, though further research into the isoform specific consequences of HNF4A targeting through endogenous ligands and small molecules requires further investigation.

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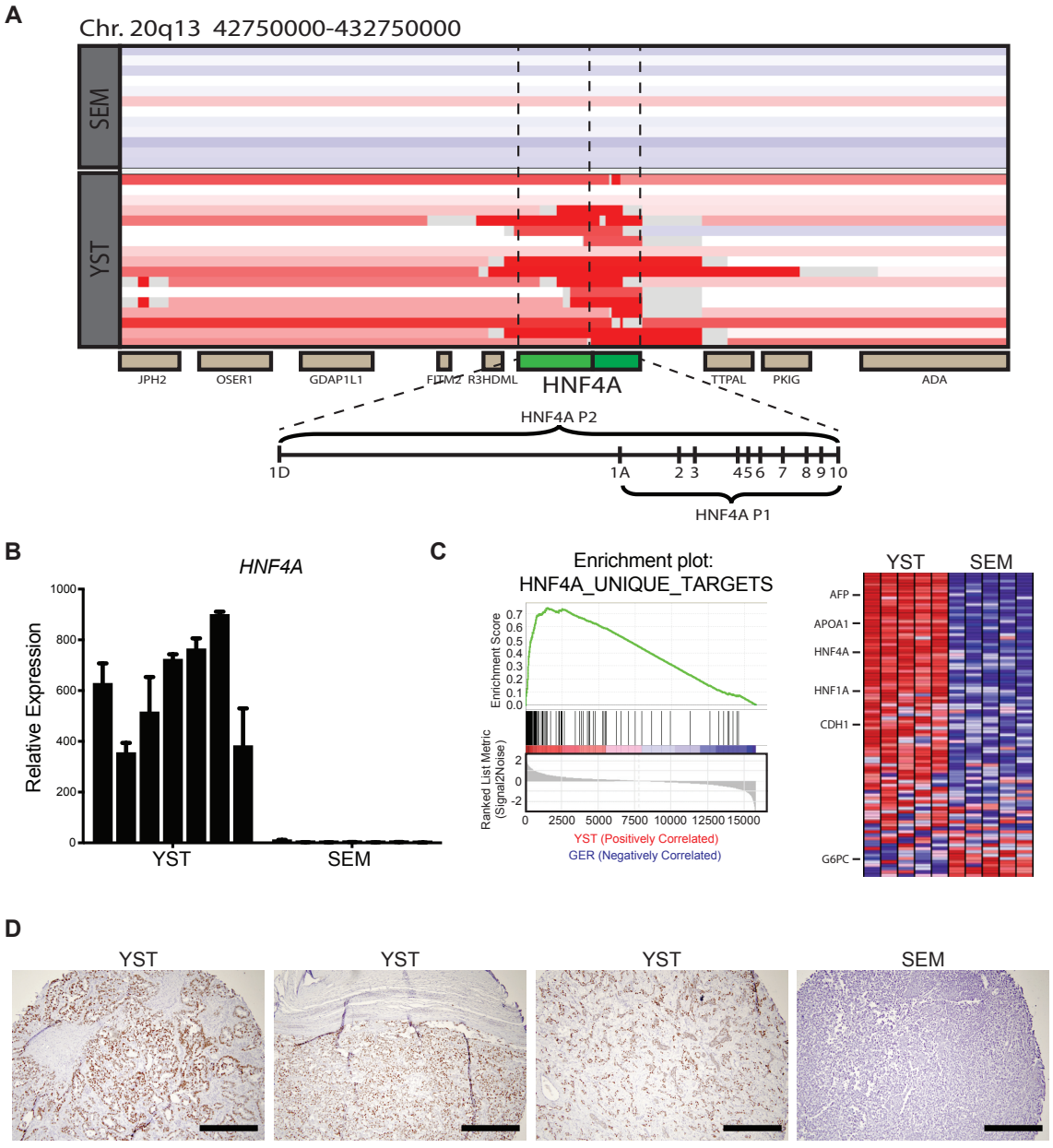
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0.6
0.5
0.4
0.3
0.2
0.1
0.0
Ranked List Metric
(Signal2Noise)
2
0
-2
0
2500
5000
7500
10000
12500
15000
YST (Positively Correlated)
GER (Negatively Correlated)
YST
SEM
AFP
APOA1
HNF4A
HNF1A
CDH1
G6PC
D
YST
YST
YST
SEM

Figure 2.1. HNF4A amplification and expression in YSTs. (A) Schematic representation of HNF4A focal copy number gains in patient YST samples. (B) Quantitative RT-PCR of HNF4A expression of 7 YSTs and 7 SEM presented as mean of three replicates +/- SD ($2^{-\Delta\Delta CT}$). (C) GSEA enrichment plot for direct HNF4A targets in YST and SEM utilizing RNA sequencing data (Left). Heat map of HNF4A target expression (Right). (E) IHC staining of YST and SEM sections stained with HNF4A general antibody H1415. Cells were counterstained with Hematoxylin.

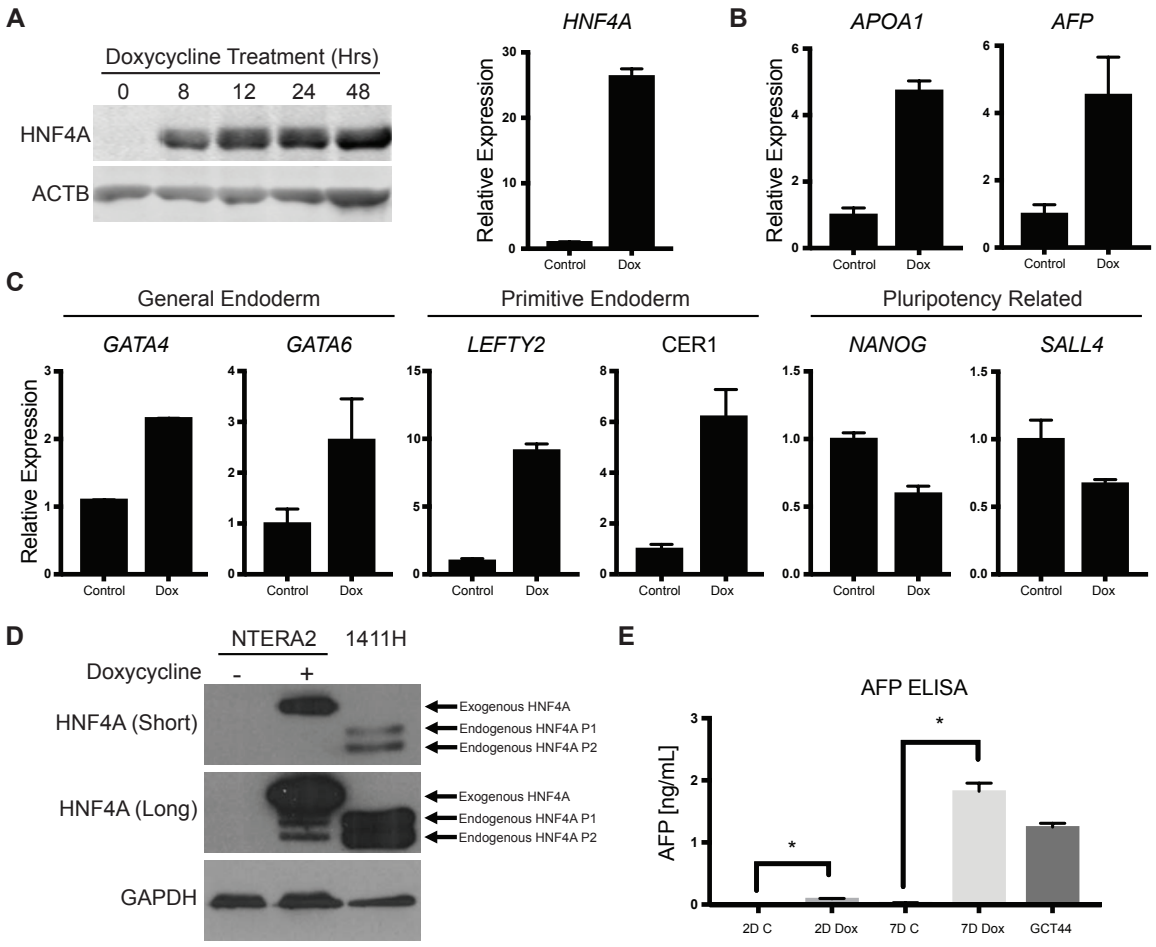
A
Doxycycline Treatment (Hrs)
0 8 12 24 48
HNF4A
ACTB
HNF4A
Relative Expression
Control Dox
B
APOA1
AFP
Relative Expression
Control Dox
C
General Endoderm
Primitive Endoderm
Pluripotency Related
GATA4
GATA6
LEFTY2
CER1
NANOG
SALL4
Relative Expression
Control Dox
D
NTERA2 1411H
Doxycycline - +
HNF4A (Short)
HNF4A (Long)
GAPDH
Exogenous HNF4A
Endogenous HNF4A P1
Endogenous HNF4A P2
E
AFP ELISA
AFP [ng/mL]
2D C
2D Dox
7D C
7D Dox
GCT44

Figure 2

Figure 2.2. HNF4A expression is sufficient to activate primitive endoderm YST gene expression in a GCT context. (A) Western blot of NTERA2 cells after doxycycline controlled induction of HNF4A at multiple time points (Left). Quantitative RT-PCR of HNF4A expression after 48hrs HNF4A induction presented as mean of three replicates +/- SD ($2^{-\otimes\otimes CT}$) (Right). (B) Quantitative RT-PCR of APOA1 and AFP expression after 48hrs HNF4A induction presented as mean of three replicates +/- SD ($2^{-\otimes\otimes CT}$). (C) Quantitative RT-PCR of multiple primitive endoderm markers at 7 days of HNF4A induction presented as mean of three replicates +/- SD ($2^{-\otimes\otimes CT}$). (D) Immunoblots for HNF4A and GAPDH in NTERA2 cells after 7 days of HNF4A induction. YST cell line 1411H used for comparison of endogenous HNF4A. (E) Secreted AFP production following 2 days and 7 days of HNF4A induction compared to the AFP secretion of YST cell Line GCT44. As measured by AFP ELISA.

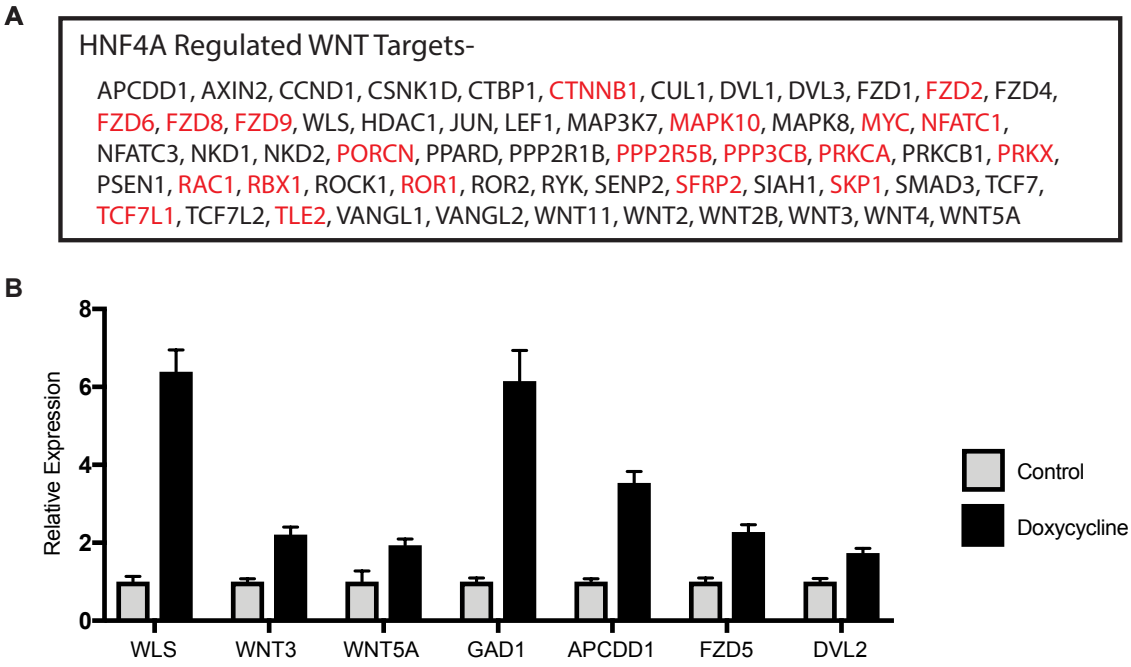

Figure 2.3. HNF4A expression modulates the WNT pathway in GCTs. (A) Heat map demonstrating the WNT pathway gene expression changes in NTERA2 cells following induction of HNF4A for 7 days. (B) Quantitative RT-PCR of selected WNT pathway genes after 7 days of HNF4A induction ($2^{-\Delta\Delta CT}$).

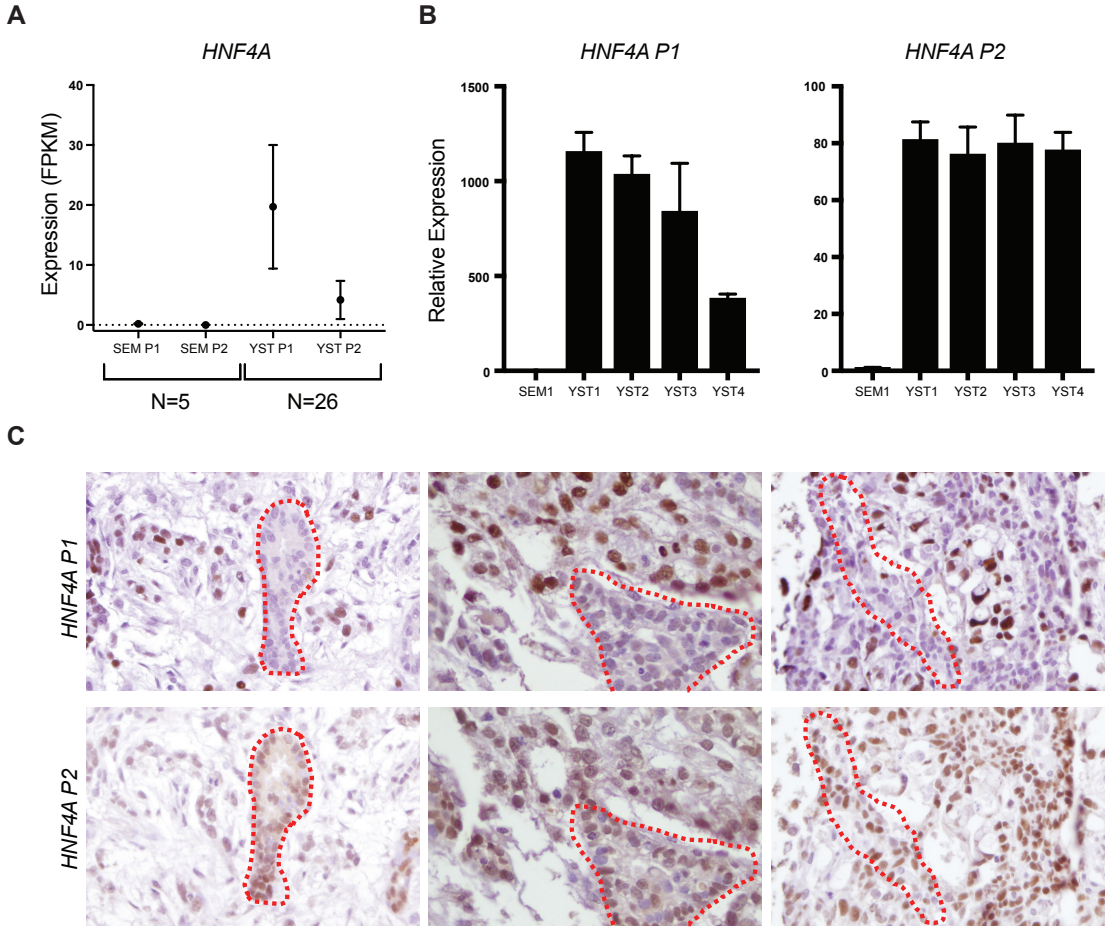

Figure 2.4. Human YST samples exhibit robust expression of HNF4A isoforms with distinct intra-tumoral localization. (A) Average FPKM of HNF4A isoforms from multiple YSTs compared to SEM. (B) Quantitative RT-PCR of YST and SEM patient samples for HNF4A P1 and HNF4A P2 ($2^{-\Delta\Delta CT}$). (C) Immunohistochemical staining for P1 HNF4A (Upper three panels) along with a step section stain for P2 HNF4A (Lower three panels). Red dotted line highlights regions with varying cell morphology and isoform specific expression.

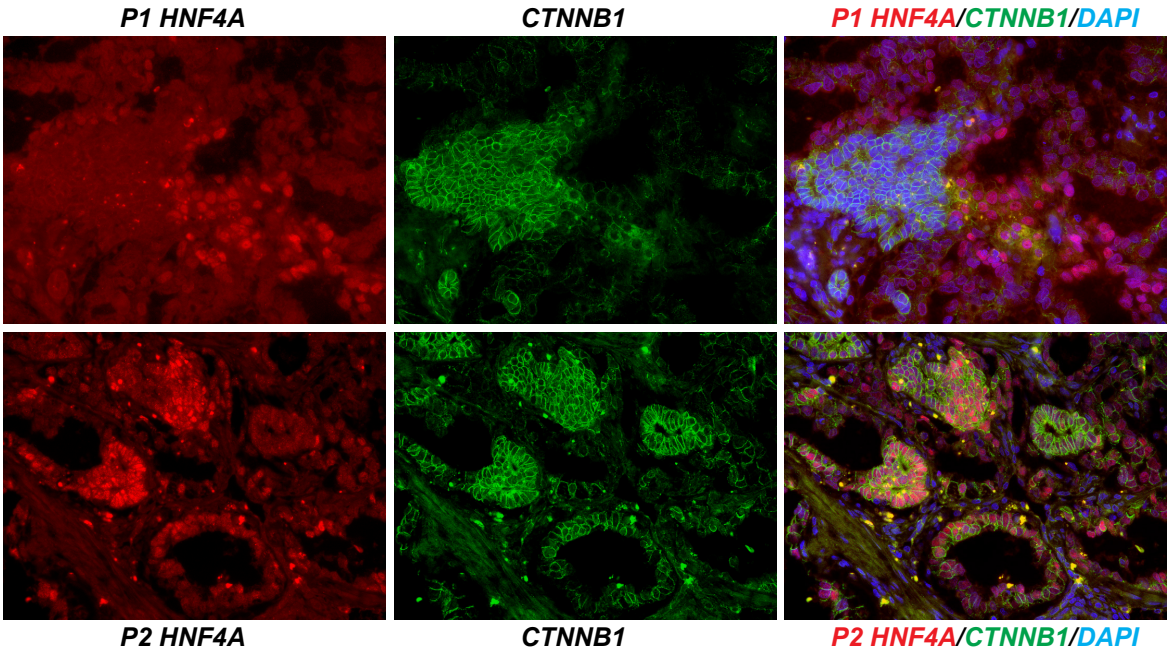

Figure 2.5. Immunofluorescent staining of YST for HNF4A P1/P2 and CTNNB1. The stains are CTNNB1 (Red), HNF4A P1 (Top)/ HNF4A P2 (Bottom) (Green), and DAPI (Blue). Top panels exhibit HNF4A P1/ CTNNB1 localization which reveals a correlation between HNF4A P1 expression and cytoplasmic CTNNB1. Bottom panels exhibit HNF4A P2/ CTNNB1 localization which shows a HNF4A P2 expression favoring membranous CTNNB1 in HNF4A P1 negative cells.

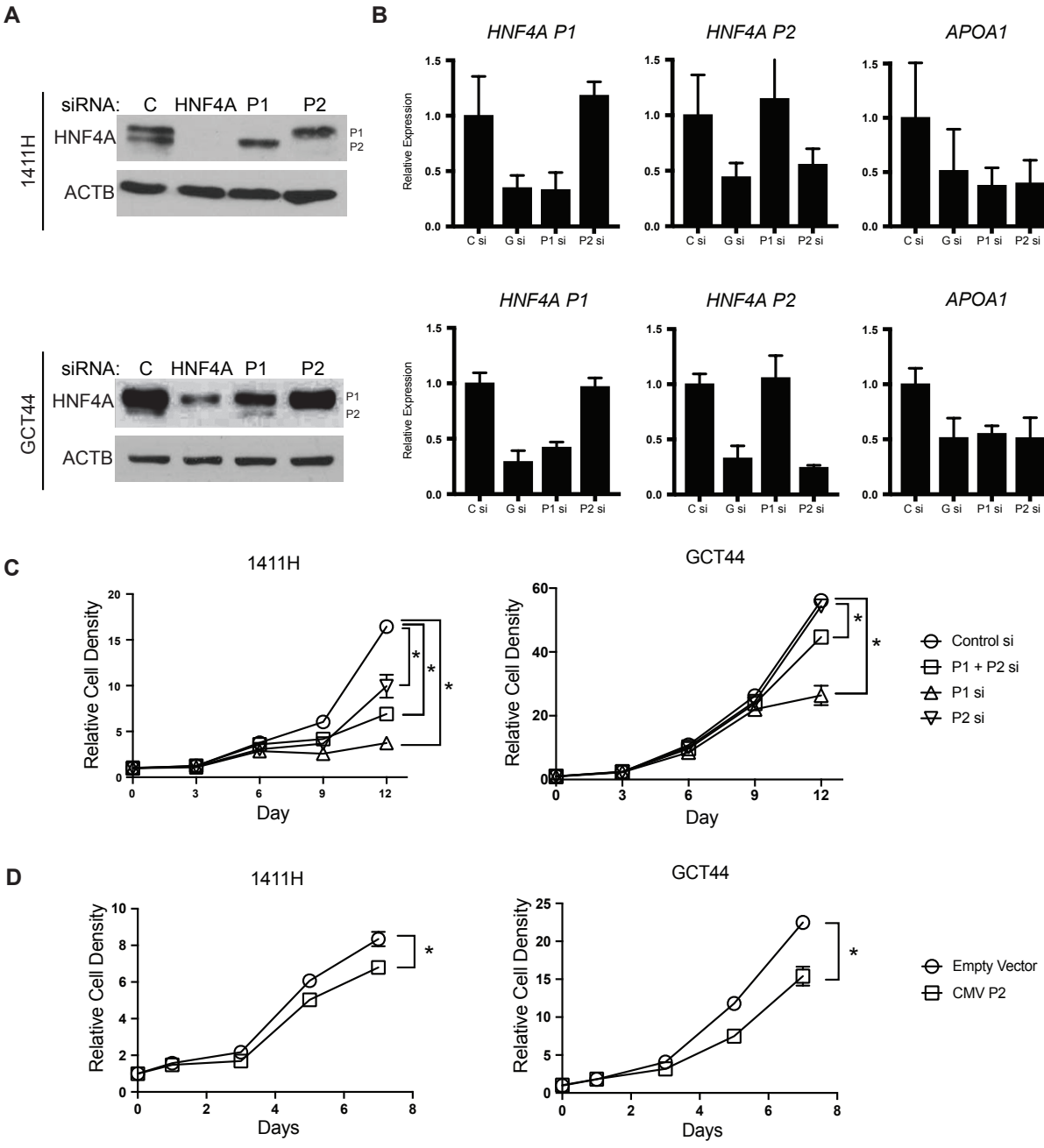
A
siRNA: C HNF4A P1 P2
1411H
HNF4A
P1
P2
ACTB
GCT44
B
HNF4A P1
HNF4A P2
APOA1
Relative Expression
C si
G si
P1 si
P2 si
C
1411H
GCT44
Relative Cell Density
Day
Control si
P1 + P2 si
P1 si
P2 si
D
Days
Empty Vector
CMV P2

Figure 2.6. HNF4A isoform specific disruption in vitro reveals susceptibility to loss of P1 isoforms. (A) Immunoblots for HNF4A and ACTB after 48hr treatment with multiple HNF4A siRNA. The top band represents the P1 isoform family and the bottom band represents the P2 isoform family. (B) Quantitative RT-PCR of YST line cell lysate after HNF4A siRNA treatment using HNF4A P1 and HNF4A P2 specific primers as well as primers for HNF4A target APOA1 ($2^{-\otimes\otimes CT}$). (C) Crystal violet growth assay on YST cell lines treated with different HNF4A siRNA (* = P<.05). siRNA treatment was repeated every three days to maintain reduction of HNF4A protein levels. (D) Crystal violet growth assay on YST cell lines transfected with HNF4A P2 under the control of a CMV promoter (* = P< 0.05).

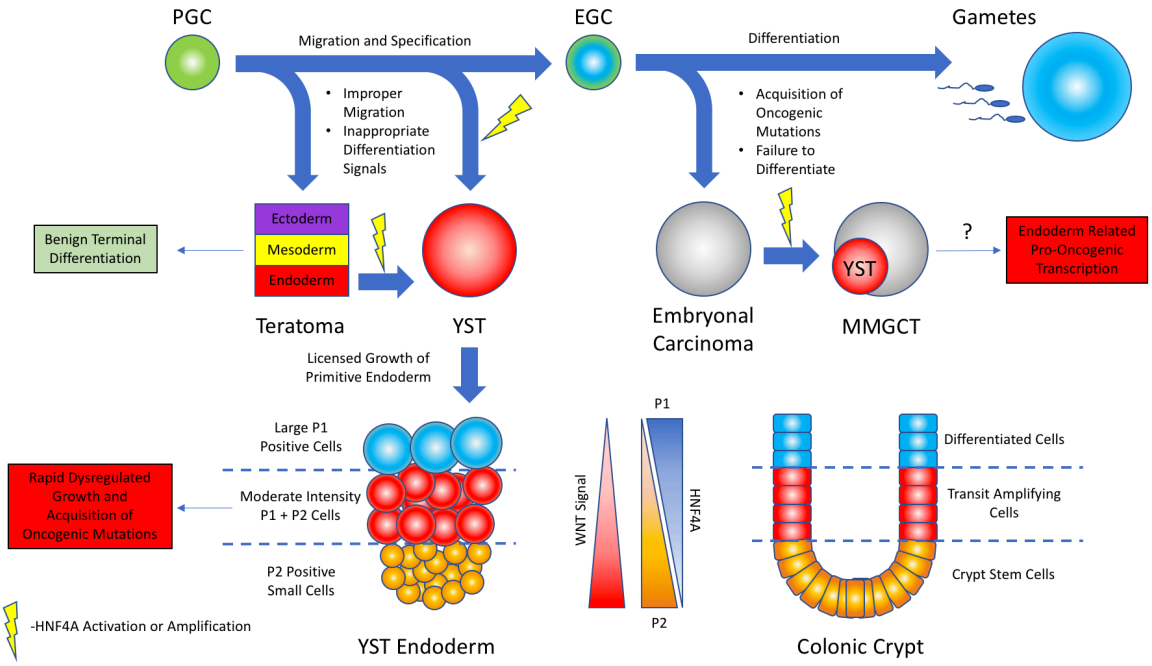

Figure 2.7. Model of HNF4A mediated YST development.

Chapter III

Pediatric Germ Cell Tumors as a Developmental Disease: Vulnerabilities in Primordial Germ Cells

Introduction

Amongst the myriad and heterogeneous neoplastic malignancies presented to the oncologist, the prevailing conception of tumorigenesis has remained relatively static. Somatic "Cells-of-Origin" acquire genetic lesions that result in clonal growth and subsequent rounds of proliferation select for clones of highest oncogenic potential (Hanahan and Weinberg 2011). These proliferative masses assume the form of a neoplasm which confers a disease phenotype to the patient. Though this cycle does represent the common timeline for tumorigenesis in adults, biology of childhood cancers may be driven by different mechanisms, owing to the unique milieu of the developing human body. The pediatric patient possesses both immature cells and developmental signaling that may be completely absent in adult patients. This distinction holds within it both opportunity and the need for caution. It is likely the case that some pediatric malignancies are result of early developmental conditions in which misdifferentiated cells may grow out of control. Therefore, our basic biological understanding of the developmental processes involved in the tissue of origin may be invaluable to our understanding of these diseases and how to best treat them. A perfect example of this type of system is that of pediatric germ cell tumors (pGCTs) and their presumptive precursor, the primordial germ cell (PGC), the

study of which has provided a much broader understanding of the etiology of this heterogeneous and prevalent disease. Our goal is to

describe the current state of the field with regard to the developmental and molecular mechanism underlying GCT development as well as offering new insights.

The PGC is responsible for specification of the germline and its responsibility for maintaining the pluripotent potential necessary for gamete generation involves a unique developmental cycle which involves stages of vulnerability to improper differentiation. The PGC must be specified from the rest of the developing embryo through genetic and epigenetic events; it subsequently migrates throughout the body to the site of the gonad; and it must then undergo sex specific differentiation. Each of these stages reflects both a developmental feature as well as a clue related to the phenotypes and characteristics of germ cell tumors.

PGC Specification and Pluripotency

The first event in the development of the germline is the specification of PGCs in the early embryo. This specification event endows the PGCs with pluripotency as marked by a unique histological and genetic signature. PGC specification in humans occurs at ~2 weeks post fertilization when BMP signals target to the mesendoderm of the pre-primitive streak embryo which lead to the induction of SOX17 which, with BLIMP1, suppresses endodermal differentiation and activates PGC differentiation (Tang et al. 2016). These cells express pluripotency associated markers OCT3/4, LIN28A, and NANOG histologically and begin a process of global DNA demethylation necessary for the later establishment of sex specific gametic imprinting (Leitch et al. 2010; West et al. 2009; De Felici 2011). This genetic and

epigenetic state creates the platform for development of the gamete which leads to formation of the totipotent zygote after fertilization.

This unique developmental licensing of pluripotency is likely partially responsible for the extremely heterogeneous histological variation amongst germ cell tumors. Unlike most other tumor types, germ cell tumors may present with cells from each of the three germ layers as well as undifferentiated, germline, and extraembryonic cells (Rakheja and Teot 2014). In addition to GCTs exhibiting histological heterogeneity it has been shown that both seminomas and non-seminomas retain pluripotency associated protein expression (SALL4, OCT3/4, LIN28A) which indicate the pluripotent growth potential even amongst the different differentiation states (Rakheja and Teot 2014). Furthermore, it has been shown that LIN28A expression in malignant GCTs is responsible for maintaining an undifferentiated state through repression of the tumor suppressive let-7 miRNA family (Murray et al. 2013). Additionally, the protein EPCAM, which is expressed in undifferentiated embryonic stem cells (ESCs) is associated with malignant non-seminomatous GCTs and has been suggested as a serum diagnostic marker in the treatment of GCTs (Schonberger et al. 2013; Ng et al. 2010). GWAS studies have revealed at GCT associated SNP near the gene PRDM14, a key PGC pluripotency marker in mouse (Ruark et al. 2013; Ohinata et al. 2005). Though the function of PRDM14 is still being elucidated in humans it is possible that it is involved in maintenance of the pluripotent state through the initiation of global demethylation as it is in mice (Okashita et al. 2016). The association of PRDM14 with GCT furthers the notion that GCT biology is intimately associated with PGC development rather than traditional oncogenes. The second step of PGC specification, the previously mentioned global demethylation, is another hallmark of GCTs and may be related to GCT susceptibility (Tang et

al. 2016). DNA demethylation at this stage allows for bi-allelic expression of genes (Lee et al. 2002). Research on methylation states of the gene SNRPN revealed that most GCTs possess hypo-methylation which lends credence to the idea that GCTs were derived from PGCs (Bussey et al. 2001). In addition to connecting GCTs to PGCs this loss of transcriptional control likely provides a vulnerability in GCT development as loss of imprinting is a common event in many cancers (Jelinic and Shaw 2007). This open epigenetic environment is thought to be protected by activation of the repressive chromatin modifications H3K27me3 and H3K9me3 concurrent with global demethylation, however another GWAS SNP near ATF7IP, a gene related to chromatin dynamics, may play a role in disrupting this repressive mark (Hackett et al. 2012; Turnbull et al. 2010). Disregulation of ATF7IP with its cofactor SETDB1 may interfere with proper repressive chromatin marks, as it is known to be involved in creation of H3K9me3 marks, though further work is needed (Basavapathruni et al. 2016; Ichimura et al. 2005). It is likely that the pluripotent specification of PGCs as well as the physiological loss of imprinting naturally provide two pro-oncogenic conditions that may be coopted by improper differentiation cues.

This creation and maintenance of pluripotent state prior to and during migration may explain the histologies specific to GCTs in young children which are often referred to as Type I GCTs. The type I GCTs generally present in the form of the teratoma (TER) which is generally benign, and the more problematic yolk sac tumor (YST) (Rakheja and Teot 2014). As mentioned previously, the teratoma exhibits cell structures from each of the three germ layers and can generally be treated with surgical resection. This tumor frequently presents in a mature form with fully differentiated cell types. It is likely that these tumors are the result of improper regulation of pluripotency in which reacquisition of epiblast-like pluripotency during improper PGC

specification results in cells that attempt to recapitulate embryonic development, including creation of the three germ layers. These cells are not, however, receiving the very specific spatial and temporally differentiation cues needed for proper embryogenesis which results in the disordered structure of teratomas. These tumors are likely benign because the improper differentiation is the result of a failure of development rather than the acquisition of an oncogenic mutation and therefor cease growth according to the vicissitudes of the signaling milieu in which they develop. The YST in contrast is the more rare and malignant of the type I GCTs. Old and new insights into PGC development may reveal why this tumor which is composed of one germ layer, endoderm (or two if one considers embryonic and extraembryonic endoderm separately), takes on a much more traditional oncogenic phenotype. These tumors histologically resemble many differentiated endodermal structures and possess regions of primitive endoderm (PE) histology and grow rapidly requiring treatment through surgical resection and adjuvant chemotherapy (Rakheja and Teot 2014; Nogales, Preda, and Nicolae 2012). This may be the result of two facts, firstly that PGCs are primed for endodermal differentiation owing to certain developmental aberrations and secondly that PE tissues may be licensed for growth due to lack of proper differentiation cues in children who are past the developmental stages in which these tissues exist in the body. As mentioned previously PGC specification is controlled by a concerted effort between SOX17 and BLIMP1, the second of which is responsible for preventing endodermal differentiation (Irie et al. 2015). This is required because SOX17 in the absence of BLIMP1 or even in excess of BLIMP1 is responsible for specification of the definitive endoderm (DE), a PE structure responsible, through complex differentiation programs, for the development of most adult endodermal structures (Ying et al.

2015). Kobayashi et al. recently proved, in porcine, monkey and human systems, that even after PGC priming of mesendoderm cells, overexpression of SOX17 or loss of BLIMP1 resulted in differentiation to DE (Kobayashi et al. 2017). Interestingly, almost 3 decades ago, it was shown in rats that externalization of fetal yolk sac PE after fetectomy was capable of formation of malignant YSTs which could be transplanted to syngeneic rats (Sobis, Verstuyf, and Vandeputte 1993). This provides evidence that PE cells have the capacity to grow rapidly and acquire oncogenic lesions which enhance growth when externalized from their normal developmental location. Rather than terminally differentiating like their teratoma counterparts they grow rapidly and act as the source of their own growth regulatory signals as many endodermal compartments do (Stevens et al. 2017). This proliferative licensing of PE may serve as the explanation for the GWAS SNP associated with HNF1β which is an important transcription factor related to endoderm differentiation and maintenance (Kristiansen et al. 2015). Aberrations in HNF1β signaling in the context of a PGC may favor PE mis-differentiation and subsequent YST formation. This hypothesis is in accordance with the data presented in chapter II which indicates a role for endodermal transcription factors in YST development. It may be the case that aberrant overexpression of a number of different endodermal transcription factors may lead to YST formation owning to the pluripotent licensing of PGCs.

PGC Migration and Proliferation

After the PGCs have been specified as the independent germ lineage they must make their way to the genital ridge, proliferating en route, where they will receive subsequent differentiation cues for sex specific development. However, this journey serves as a source of

developmental vulnerabilities that may explain the frequent extragonadal localization of pGCTs. After specification of the PGCs in the early mesendoderm ~2 weeks after conception they make their way to the wall of the yolk sac endoderm (Aeckerle et al. 2015). Subsequently PGCs migrate along the hindgut and midgut endoderm until near the gonadal ridge (GR) (Mamsen et al. 2012). At this point they migrate through the dorsal mesentery to the dorsal body wall where they migrate laterally to the GR. This migration generally results in the specific localization of viable PGCs to the GR, however multiple sections of this tightly controlled migratory pathway present developmental vulnerabilities.

Pediatric germ cell tumors frequently present in extragonadal locations in pediatric patients. These locations are limited to midline structures such as the sacrococcygeal, retroperitoneal, mediastinal, cervical, and intracranial regions. The migratory route coupled with the molecular mechanisms governing PGC migration are likely the causal factors in GCT localization. Though the exact mechanism by which PGC migration is controlled are not fully understood multiple cytokine mechanisms have been implicated. Interruptions of these pathways have revealed ectopic germ cell localization. The GWAS SNP most strongly correlated to GCT risk resides in the KITLG locus which is thought to though to regulate migration from the midgut to the GR though this role may be overstated in regard to GCT formation and other likely impacts of this gene will be mentioned later (Poynter et al. 2012; Chanock 2009). Knockdown of CXCR4 and its ligand CXCL12 results in PGC mis-migration and failure to reach the GR after PGCs reach the dorsal wall (Doitsidou et al. 2002). β-integrin and E-cadherin cell surface proteins as well as surface lipids have been implicated in migration along the midgut endoderm (De Felici, Scaldaferri, and Farini 2005). Finally, a study by Runyan et al. revealed that rather

than PGCs specifically migrating to the GR they actually normally mis-migrate to multiple locations and are cleared through a BAX dependent apoptotic program (Runyan et al. 2008). Intriguingly, GWAS studies found that a SNP near the BAK1 locus, which is an important member of the BCL2-BAX-BAK1 anti-apoptotic axis, was associated with GCT risk (Lindqvist and Vaux 2014). These observations together reveal the developmental vulnerabilities of PGC migration.

If perturbations of these migratory mechanisms result in failure to appropriately reach the GR we would expect to find extragonadal GCTs (EGGCTs) in body regions that develop from the tissues along which PGCs migrate and in fact for multiple regions that is the case. The germ cells must migrate past the dorsal aorta from the dorsal mesentery on the way to the GR. Migration failure at this point would result in PGCs residing along and near the aorta which eventually resides in the mediastinum and the retroperitoneum, two frequent sites of EGGCTs (Mamsen et al. 2012). Anterior-posterior, rather than lateral, mis-migration along the body wall may be the mechanism for EGGCTs occurring in the sacrococcygeal and cervical regions. Aberrant migration from the hindgut could explain sacrococcygeal GCT prevalence as this structure is caudal and adjacent to the region which will form the tailbone. Alternatively, another mechanism based on nerve fibers may explain the distal localization of some EGGCTs. Møllgård et al. found that PGCs migrate along nerve fibers from the midgut along the dorsal mesentery and into the GR. These PGCs associated intimately with the peripheral Schwann cells (Mollgard et al. 2010). These data suggest that cells of the autonomic nervous system (ANS) may be responsible for secreting cytokines which PGCs recognize as migration and proliferative cues. This idea is further corroborated by studies involving the secreted neural signaling molecules

PACAP and GDNF, both of which have migratory and proliferative effects on germ cells in vitro (Mollgard et al. 2010; Dovere et al. 2013; Pesce et al. 1996). These proteins are both secreted by the ANS and could explain the cranial, cervical, and sacrococcygeal localization of ectopic PGCs and EGGCTs as these regions are thoroughly enervated by the ANS. Based on this evidence the link between PGC migration and ANS signaling is warranted.

During the migratory phase of PGC development their numbers expand rapidly in order to properly colonize the GR. This proliferative step may be a key in the initiation of pGCTs. The primary determinant of this proliferative step is signaling through the receptor tyrosine kinase (RTK) KIT. As PGCs migrate from the midgut to the GR somatic KIT ligand (KITLG) secretion induces rapid PGC proliferation and suppresses apoptotic pathways used to clear ectopic cells (Mahakali Zama, Hudson, and Bedell 2005). The signaling axis relating to KIT controlled proliferation has proven especially interesting due to the fact that two primary interactors have been discovered to have associated SNPs linked to GCT risk. SNP variants near KITLG have revealed a GCT associated odds ratio of approximately 2.5 which represents the strongest GCT SNP association to date and one of the highest general cancer associations ever (Chanock 2009; Hersmus et al. 2012; Poynter et al. 2012). Mahakali et al. showed through a serious of mutations to the KITL (murine KITLG homolog) that interruption of KITLG signaling resulted in diminished PGC proliferation (Mahakali Zama, Hudson, and Bedell 2005). In vitro assays have shown that excess KITLG can induce rapid proliferation of PGCs (Moe-Behrens et al. 2003). Human studies of KIT and KITLG SNPs have shown association with low levels of fertility (Galan et al. 2006). It is possible that the KITLG variations which cause a high predisposition to GCTs remains in the population through elevation of fertility. The KITLG association with

GCTs is likely due to a variation which causes some increase in KIT signaling which results in more robust PGC proliferation. This expansion of the pool would create two independent vulnerabilities to GCT development. First the rapidly dividing pool of cells is vulnerable to acquisition of mutations that may drive oncogenesis and becomes doubly so when proliferation is increased through excess KITLG secretion. Secondly KIT signaling activates pro-survival pathways that would allow for expansion of cells that have acquired genetic lesions as well as survival of ectopic PGCs if the range of KITLG secretion was increased by the SNP variants. Further evidence strengthening the relationship of the KIT axis to GCTs is the discovery that a SNP near the gene SPRY4 is associated with GCT risk (Poynter et al. 2012). SPRY4 is an inhibitor of RTK signaling and has been show to abrogate KIT signaling (Felfly and Klein 2013; Cabrita and Christofori 2008). A SNP at this locus resulting in reduced SPRY4 expression could serve a function similar to KITLG increase by failing to reduce KIT signaling. It has also been found that KIT is frequently mutated in intracranial germinomas, however that finding may be an independent mechanism that recapitulates RAS signaling in Type II GCTs rather than an aberration of normal developmental pathways as would be the case in KITLG mediated PGC proliferation (Kamakura et al. 2006). This would explain why KITLG is associated with GCT risk for all histologies while KIT mutations are limited to germinomas. These results suggest that pGCTs are the result of slight aberrations in normal developmental pathways.

Germline Differentiation

Upon arrival to the nascent gonad the PGCs must begin the transition from migratory pluripotent cells into either sperm or egg progenitors. This process is controlled through

induction of sex specific gene expression mediated by ligand secretion from the somatic cells of the gonad as well as circulating hormones. The set of developmental processes responsible for sex determination are tightly controlled and interruption of these differentiation mechanisms represent the final set of vulnerabilities to pGCT development. Furthermore, work from the Looijenga group has carefully laid out the case that failure of sexual development in the context of disorders of sex development (DSDs) are the proximate cause of the additional GCT risk observed in these patients (Looijenga, Van Agthoven, and Biermann 2013). I believe that this observation along with other noted characteristics of pGCTs in the developing gonad further reinforce my hypothesis that most pGCTs are the end phenotype of PGC developmental defect.

In the context of gonadal pGCTs and more frequently in GCTs from older patients a precursor lesion known as germ cell neoplasia *in situ* (GCNIS) is found in otherwise normal gonadal tissue adjacent to tumor tissue (Berney et al. 2016). These lesions exhibit histological markers, methylation status, and gene expression profiles that are remarkably similar to PGCs, which should normally be absent at the developmental stages at which GCNIS is observed (Stoop et al. 2008). It is likely that GCNIS represents an aberrant uncontrolled PGC proliferation controlled either by failure of differentiation cues in pGCTs or the acquisition of oncogenic mutations such as KRAS or KIT in the adult GCTs, though in some cases these effectors may be the cause of the lesion in other age ranges. These PGCs or GCNIS have been shown to exist in a metastable differentiation condition *in vitro* in which a truly naïve pluripotent cell known as an embryonic germ cell (EGC) can be derived (Kimura and Nakano 2011). EGCs exhibit epiblast pluripotency marks such as OCT3/4 and SOX2 and are capable of teratoma formation (Attari et al. 2014). Additionally, newly discovered SNP variants of embryonic pluripotency related genes

ZFP42 and TFCP2L1 have been associated with GCT risk (Tang et al. 2015; Scotland et al. 2009). This observation that a precursor primitive germ cell derivative exists in a metastable differentiation state with an embryonic state, coupled with the aforementioned predisposition to PE differentiation mediated by SOX17, creates a straightforward model for understanding how GCTs can present with such varied histology (Figure 2.1). My hypothesis is that an OCT3/4-SOX2-SOX17-BLIMP1 network controls the differentiation state of pGCTs, with interruption of different factors by aberrant development responsible for differential histologies.

The unique susceptibility of PGCs to aberrant differentiation may explain the variety of genes implicated in GCT GWAS studies. Rather than traditional SNPs involved in pro-oncogenic or tumor suppressive activities, such as MYC or CDKN1A in colorectal cancer (Kang et al. 2015; Dai et al. 2012), GCT SNPs may cause slight perturbations in PGC development that lead to differentiation failure, mis-migration, or aberrant proliferation. This hypothesis is evidenced by multiple mouse models in which the loss of genes responsible for PGC maintenance and eventual sexual differentiation are interrupted and pGCTs result. Perhaps the most famous mouse pGCT model is the 129/Sv inbred mouse strain in which mutations in the Dnd1 gene result in spontaneous teratoma formation (Noguchi and Noguchi 1985; Youngren et al. 2005). Two sentinel GWAS loci near the genes DAZL and DMRT1, which are responsible for specification of the germline during sexual development, have also been implicated in teratoma susceptibility (Ruark et al. 2013; Poynter et al. 2012). Loss of any of these three genes releases suppression of proliferation and pluripotency in PGCs (Chen et al. 2014; Krentz et al. 2009).

Conclusions

Pediatric germ cell tumors are a unique group of malignancies in the scope of children's cancer. A unique combination of varied histology, wide range of sites of presentation, and lack of traditional oncogenic insults suggest an etiology reliant on mechanisms different from that of adult cancers. Additionally, germ cell tumors in general represent the only non-somatic tumor lineage in the body. I argue that these seemingly disparate factors can be linked through an understanding of normal and abnormal developmental pathways experienced by the cell-of-origin, the PGC. Furthermore, I argue that pGCTs are the downstream phenotype of aberrant PGC development rather than the result of the traditional adult tumor development model. This argument is based on the fact that the very specific biological processes necessary to propagate the species and retain totipotency provided a unique set of vulnerabilities to the development of neoplastic tissue as opposed to the slow transition to oncogenic fate through the acquisition of genetic lesions. These more likely represent aberrant differentiation that in the case of YSTs and EC lack appropriate terminal differentiation cues and thus grow unchecked. This unchecked growth then provides the possibility for normal neoplastic development.

For this reason, further study into the mechanisms of normal PGC development are warranted. The more we can understand the susceptibilities that exist within that developmental lineage the more vigilant we can be in the early detection of these lesions. New diagnostic and prognostic markers may allow for removal of pretumorous nests of aberrantly migrated PGCs. Moreover, expanding our knowledge of proper PGC development will elucidate the mechanisms by which difficult to interpret GWAS loci may play a role in the aberrant development and oncogenesis as has been the case with genes like DAZL and DMRT1.

With regard to treatment of existing pGCTs, which currently relies on surgical resection and non-specific chemotherapeutic agents, I believe that our model of abnormal PGC based etiology suggests new directions for the development of targeted treatment methodologies. These tumors that present as either benign teratomas, which in our model represent dysregulated somatic terminal differentiation, and yolk sac tumors, which represent primitive endodermal tissue with lack of terminal differentiation. The common theme amongst these most common histologies (and rare histologies such as EC or SEM) is a failure to either respond appropriately to differentiation cues or a complete lack of germ layer specific differentiation cues, which is likely the case with the extraembryonic differentiation of YSTs. Therefore, differentiation based therapies using either exogenous signaling ligands or small molecule activators or inhibits may provide a powerful mechanism for abrogating the rapid growth phenotype in these tumors. For example, it has been shown that non-seminomatous GCTs exhibit activation of WNT signaling histologically which is likely representative of the endogenous WNT signaling present in the tissue types mimicked by those histologies. It is possible that clinically approved WNT inhibitor drugs may be useful in treating these tumors by providing appropriate differentiation cues.

I believe that this developmental disease model in pediatric cancer will carry over into multiple pediatric malignancies such as Wilms tumor or rhabdomyosarcoma in which the cell of origin is likely to be a transient cell type that only exists during fetal or childhood development. Therefore, I believe that understanding the complex developmental processes involved in tissues of origin for pediatric tumors will key in developing targeted molecular therapeutics for these diseases. Additionally, the complex developmental milieu of these diseases favors in vivo model systems which can recapitulate the endogenous environmental cues of these cells-of-origin.

Pediatric cancer research using this developmental model will have the two-fold effect of elucidating the mechanisms of normal development as well as providing novel insights into pediatric cancer susceptibility.

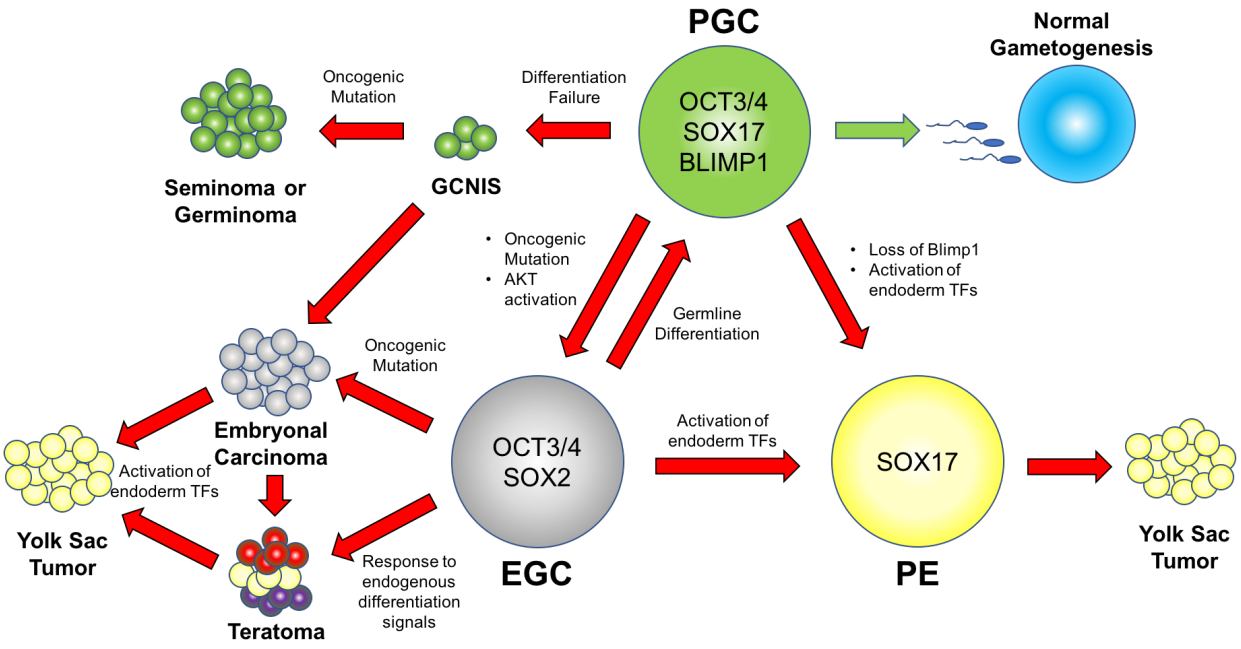

Figure 3.1. Primordial Germ Cell Vulnerability Based Germ Cell Tumor Development Model.

Chapter IV

Future Directions

Our model for understanding differentiation based oncogenesis in germ cell tumors opens multiple avenues for further study. First of all, based on the conclusion that these tumors are disorganized and primitive attempts at differentiation then investigation into potential therapeutic strategies that may drive terminal differentiation or prevent differentiation to proliferative lineages is warranted. In the context of the WNT active yolk sac tumor it may be possible to use WNT inhibitors to prevent proliferation and improve relapse free survival after surgical resection. Along the same lines it is possible that indirect WNT antagonistic treatments may be possible such as BMP activation through exogenous ligand or small molecule based therapies. Much as BMP drives terminal differentiation in GI tissues it may curb proliferation in a YST. Secondly, further investigation into isoform specific cell types and gene regulation in YSTs will likely reveal new targets. In our data targeting of P1 isoforms was more efficacious in slowing YST growth. Therefore, it follows that there are some isoform specific transcriptional targets that will prove as important regulators of YST growth. This deeper understanding of isoform specific cell type regulation may be achieved through techniques such as RNA-seq of isoform specific cell types after laser capture microdissection (LCM). It may also be necessary to perform isoform specific CHIP-seq of HNF4A and CTNNB1 to learn about the interactions of these TFs in YSTs.

Additionally, there may be fertile ground to explore with regard to learning about other endodermal molecular pathways that promote this lineage as oncogenic in the context of GCTs. In children, it is exceedingly rare to find GCTs of non-endoderm origin and the primary concern in removing a teratoma is that the endodermal portion might acquire malignant potential. Are there some characteristics of endoderm that lend themselves to malignancy or is this licensing of growth based on the fact that YSTs can resemble extraembryonic endoderm? The first case could lead to revelations that would increase our knowledge about the oncogenic potential of multiple endodermal systems. The second case could reveal unique vulnerabilities that YSTs may hold, such as expression of fetal proteins that provide oncogenic potential, the targeting of which could provide exquisitely tumor specific therapies as these proteins are not required in the body after birth.

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