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Teratomas Derived from Embryonic Stem Cells as Models for Embryonic Development, Disease, and Tumorigenesis

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1. Introduction

The word teratoma is derived from the Greek *teratos* which means “deformity” or “monster” and *-oma* which means “tumor”. Thus, the teratoma is a monster tumor. A more recent definition which is more elaborate and perhaps more politically correct is an encapsulated tumor with tissue or organ components that can be traced to derivatives of the three primordial germ layers; ectoderm, mesoderm, and endoderm (Rosai and Ackerman 2004). It is easy to understand why this tumor was coined “monster” since macroscopically teratomas can appear as a conglomeration of tissue with different colors and textures some of which may be recognizable as gross anatomical structures such as hair, teeth, and limbs. The most extreme examples actually resemble a distorted fetus (fetiform). The initial descriptions of sacrococcygeal teratomas date back to ancient tablets written by Egyptian fetoscopists around 2500 B.C. These oddities were often attributed great significance when interpreted in concert with the concurrent cultural and religious beliefs. For example, ancient Egyptians interpreted a third foot in the middle as signifying great prosperity for the land (Oosterhuis et al. 2007). These cases likely represented rare sacrococcygeal teratomas containing a fully formed foot which has been reported in the modern medical literature (Legbo, Opara, and Legbo 2008). Seventeenth century illustrations demonstrate hair sprouting from a teratoma and at that time teratomas were thought to arise from nightmares or witchcraft while in the 19th century they were thought to arise from perverse sexual practices (Oosterhuis et al. 2007; Gatcombe et al. 2004). More recent (late nineteenth and early twentieth century) theories invoke cell biology principles purporting the derivation of teratomas from primitive germ cells. Indeed some current definitions will expand the above general definition to include their histogenesis from pluripotent stem cells (Oosterhuis et al. 2007).

While at first glance, most teratomas microscopically appear as disorganized masses with recognizable tissue types contributed from the three basic germ layers, very little is really known about their composition and how they form and this information may hold

important clues to normal and abnormal development. Studying the cellular and tissue milieu within teratomas both in vivo and ex vivo would help in beginning to answer several important questions that we will address in the following sections. With this in mind, the objectives for this chapter are:

Derivation of teratomas from stem cells and factors that influence their growth and development: This section will address the tumorigenicity of embryonic stem cells and their relationship to cancer cells, some reasons why experimental teratomas have not been a hot focus of investigation, and factors that influence experimental teratoma formation (stem cell phenotype [e.g. embryonic, induced pluripotent stem cells (ES cells, iPS cells)], genetic differences in stem cell lines, cellular and metabolic factors (e.g. mitochondrial metabolism), nature of the host where the teratoma grows, and site of injection (microenvironment)).

Current technology for tracking stem cell fate, monitoring teratoma formation, and delineation of specific tissue types in vivo: Ensuring embryonic cell transplantation safety by non-invasive in vivo detection and monitoring is a pressing need if ES cells will be used in the clinic. This section will review current methodologies for imaging of teratomas both in vivo and ex vivo. We will present some of our data using high-resolution MRI to quantify and delineate specific tissue types within teratomas.

Histopathology of teratomas: This section will discuss the myriad of tissues present in teratomas and their significance. An overview of the most common tissue types, tissues rarely found, and primitive organs seen microscopically in experimentally derived teratomas will be presented.

Teratomas in the study of embryonic development: We have examined teratomas derived from mouse, non-human primate, and human embryonic stem cells and have observed similarities and differences in quantities of tissue types derived from the three germ layers that in all likelihood have developmental implications across species. We will discuss our methods for semiquantifying specific tissue types from serial histological sections. We will present some data resulting from collaborative efforts with biomedical engineers at Carnegie Mellon University on the automated identification and quantification of tissue types from digital images of histological sections of teratomas. This section will also discuss the use of teratomas as potential models for providing insight into the molecular and genetic mechanisms of development and tissue lineage commitment.

Teratomas as models of disease: This section will outline how teratomas may be used as sources of tissue for studying specific diseases. Disease specific genetic alterations of ES cells allow for production of tissue that can recapitulate in vivo diseased tissue/organs. We will discuss the use of teratomas as platforms for studying the toxic effects of compounds/molecules/intrauterine environment on embryonic development. We present some of our recent data using ES cells as a model for Alzheimer's disease.

Teratomas in the study of tumorigenesis: This section will address the recent novel unifying theory of teratoma, germ cell tumor, and other tumor formation. We also introduce teratomas as substitute platforms for studying tumor growth and behavior.

2. Derivation of teratomas from stem cells and factors that influence their growth and development

2.1 Roots of the monster: Embryonic stem cells and cancer cells: One in the same?

In the experimental setting, teratomas can be derived from several cell types including embryonic stem cells (ES cells), embryonic carcinoma cells (EC cells), embryonic and

primordial germ cells (PGCs), and induced pluripotent cells (iPS cells) (Aleckovic and Simon 2008; Blum and Benvenisty 2008, 2009; Kooreman and Wu). Embryonic stem cells share phenotypic and genetic characteristics of cancer cells including the ability for self renewal, prolonged proliferation in vitro, lack of contact inhibition, telomerase activity and the ability to invoke angiogenesis. However, human ES cells (hES cells) are karyotypically normal (i.e. diploid), often a major difference between cancer cells. In order to maintain their ability to self renew and perpetuate indefinitely, they require special culture conditions (specialized feeder cells or conditioned media on feeder free substrates such as matrigel). HES cells have certain molecular and genetic features used to characterize them including genetic and immunohistochemical expression of pluripotency transcription factors Nanog, Oct4, and Sox2, and expression of surface antigens SSEA3, SSEA4, TRA-1-60, TRA-1-81. The triumvirate of Nanog, Oct4, and Sox2 act to promote expression of self-renewal genes while repressing differentiation genes. Loss of Oct4 results in failure of the inner cell mass to develop; while loss of Sox2 and Nanog results in defective/lack of epiblast formation and subsequent differentiation towards trophectoderm and primitive endoderm. Several genes used to generate iPS cells are also linked to cancer. These include Oct4 and Sox2 which in combination with either Nanog and Lin28 or Myc and Klf4 can transform the somatic cell back to pluripotency. (Blum and Benvenisty 2008; Chambers and Tomlinson 2009; Knoepfler 2009; Kooreman and Wu ; Lanza 2006; Loh, Ng, and Ng 2008).

ES cells have unique epigenetic features. They maintain unique demethylated CpG islands allowing for increased expression of genes compared to differentiated cells. A similar phenomenon is present in cancer cells where a small reduction in methylation is sufficient to induce cancer and conversely many cancers show hypermethylation leading to inhibition of gene expression. Many tumor suppressor genes are also hypermethylated and silenced in hES cells. Certain onco-fetal genes such as survivin (BIRC5) are enhanced in undifferentiated hES cells and teratomas derived from them. Deletion of survivin promotes apoptosis in cultured hES cells and in the teratomas derived from them. Survivin is a protein which imparts anti-apoptotic activity and may be involved in protecting cells in harsh environments and is expressed in many cancers. The anti-apoptotic activity of survivin is generated by inhibition of caspase activity. Caspases have a key role in destructing Nanog implying that survivin by inhibiting caspase activity may have a role in hES cell self-renewal. These findings suggest that untransformed hES cells are possibly tumorigenic since they already have genetic hallmarks of tumors despite having no mutational transformation. (Blum and Benvenisty 2008, 2009; Chambers and Tomlinson 2009; Knoepfler 2009; Kooreman and Wu ; Loh, Ng, and Ng 2008; Yu and Thomson 2008).

2.2 Teratomas: The reviled monster

Since first derived from the blastocyst of the developing mouse by Martin Evans and Mathew Kaufman at Cambridge and Gail Martin at UCSF independently in 1981, ES cell lines have been derived from non-human primates (1995) and then from humans by James Thomson in 1998 at the University of Wisconsin (Yu and Thomson 2008). In the mouse the same cells that produce teratomas in the experimental setting when placed back into the developing blastocyst (the blastocyst complementation assay) integrate into the embryo without tumor production and contribute to the formation of tissues derived from the three primordial germ layers (by definition pluripotent) and also the germ line. In fact, this is the test of pluripotency for potential mouse ES cells (mES cells)-the ability to form a chimera (Prelle, Zink, and Wolf 2002). Primarily due to ethical restraints, the chimera test of

pluripotency is not the method used to assess whether given cells are pluripotent for non-human primate and human cells. The gold standard has been the ability of a given collection of cells to form teratomas when injected into immunodeficient mice. This test and the production of this tumor has produced great consternation in the evolving world of regenerative medicine for several reasons that predominantly include time and cost (Dolgin). The main goal of regenerative medicine thus far has been to guide stem cells (whatever their origin) to differentiate into specific tissue types (e.g. cartilage, islets, heart muscle) to be used as new tissue for repair of injured or aging tissue. The teratoma is the antithesis of this philosophy; once again living up in a more figurative sense to its word origin roots--a monster!

Teratoma formation by stem cells remains the last frontier to conquer before effective clinical trials using stem cells can be widely accepted. Already several examples exist of the clinical use of stem cells in animals resulting in teratoma formation (Knoepfler 2009). Cells for clinical use can be grown and issues concerning immune rejection of cells have been alleviated due to the emergence of iPS cells, cells derived from nuclear transfer, and human Wharton jelly stem cells (Menendez, Bueno, and Wang 2006). Several papers have addressed specific measures developed at the genetic and cell sorting level to combat this undesirable "side effect" if you will (Koch, Jordan, and Platt 2006; Kooreman and Wu ; Cao et al. 2007; Bulic-Jakus et al. 2006). Recently, some sentiment has arisen amongst stem cell investigators to abandon the teratoma assay as the measure of pluripotency in favor of molecular/bioinformatics approaches. In Dolgin's recent article, Owen Witte, Director of the Broad Stem Cell Research Center at UCLA calls the teratoma assay; "the most ridiculous assay on the planet".

Most literature centered on teratomas derived experimentally from embryonic stem cells portrays teratomas as a means to an end or as an unwanted side effect in the clinic. This may explain the relative paucity of literature that specifically examines the factors that influence teratoma development from stem cells in the experimental setting. However, the specific articles that have focused on the teratoma shed valuable attention on how specific variables seem to affect the growth and characteristics of teratomas.

2.3 Stem cell phenotype

A single paper comparing the rates of teratoma formation using several embryonic stem cell types known to produce teratomas (e.g. mES, non-human primate (nhpES), hES cells) while controlling for variables such as site of injection, number of cells injected, phenotype of the host has not been published. The paper by Hentze et al comes close as it includes several hES cell lines, injection sites, cell numbers, and included semiquantitative histopathological analysis of the teratomas (Hentze et al. 2009). In a brief report, Gutierrez-Aranda et al (Gutierrez-Aranda et al.) demonstrated that human iPS cells produced teratomas sooner and at a higher rate per injection than human embryonic stem cells. In their study they used seven different hES cell lines and 4 iPS cell lines. They performed subcutaneous or intratesticular injections of 1×10^6 cells suspended in 30% matrigel. All mice (100%) injected with iPS cells developed teratomas regardless of injection site compared to 81% and 94% efficiency of teratoma production for subcutaneous and intratesticular injection of hES cells respectively. In addition, iPS cells developed tumors much more quickly at both sites of injection compared to hES cells. These authors claimed no differences in histological composition of teratomas based on site of injection. However, specific notation of any

histological differences in teratomas when compared between cell types (hES and iPS cells) was not made but presumably was not different. Histological analysis was defined by microscopic morphology and immunohistochemical markers of specific tissue lineages, but was not quantified. They did not report any teratocarcinomas or islands of undifferentiated cells in any of their teratomas. In our empiric evaluation of several teratomas (Castro and Ozolek unpublished observations) derived from iPS cells we have noted significant areas of undifferentiated, malignant appearing cells that would qualify the lesions as teratocarcinomas. The karyotype of the iPS cells used to derive the teratomas that we have examined was not available. Differences in mitochondrial metabolism within undifferentiated embryonic stem cells may also influence their ultimate fate. Schieke et al demonstrated that mES cells with the highest resting mitochondrial membrane potential had high metabolic rates, high resting oxygen consumption, lower rates of mesodermal differentiation in the absence of pluripotency culture conditions, and produced larger teratomas by weight compared to genetically identical mES cells with the lowest resting mitochondrial membrane potentials. Rapamycin treatment to inhibit mTOR activity produced the same results as the population of mES cells with the lowest resting mitochondrial membrane potential indicating a direct effect of rapamycin on mitochondrial function. The authors speculate that since mitochondrial metabolism is directly related to the production of reactive oxygen species and genomic instability, stem cell populations within organisms might show preferential selection of stem cells with low metabolism as a means to prevent deleterious genomic events over the lifespan of the organism (Schieke et al. 2008). This concept is important and may have implications for tumor development in humans as we will discuss in section 7.

2.4 Genetic aberrations of stem cell lines and teratoma formation

In the above article by Gutierrez-Aranda, they speculate the difference in teratoma production efficiency and latency between iPS and hES cells was due to subtle genetic differences not able to be detected by conventional G-banding karyotyping and that more sensitive techniques would need to be employed to answer this question (Gutierrez-Aranda et al.). Two of their lines (one iPS and one hES cell line) were aneuploid, but apparently did not show any differences in the histological composition of teratomas derived from them compared to euploid cell lines. The aneuploidy was not further specified. HES cells can undergo chromosomal derangements when cultured for extended periods. The most common aberrations noted include gains of chromosomes 12, 17, and X but others have been noted (Blum and Benvenisty 2008). However few studies have shown or addressed the issue of teratoma production from stem cells known to harbor genetic aberrations. An interesting paper by Karin Gertow et al at the Karolinska Institute from 2007 demonstrated hES cells with trisomy 12 derived from the HS181 line in culture maintained under pluripotent conditions (Gertow et al. 2007). HES cells with trisomy 12 were found intermingled with diploid cells or could be found as the predominant cell type in some colonies. Several interesting results from this paper: The percentage of trisomy 12 cells in culture increased with passage number. The overall amount of tissues present in teratomas was roughly the same between teratomas derived from predominantly diploid versus predominantly trisomy 12 hES cells with the exception of increased renal tissue in trisomy 12 teratomas. The authors postulated that since approximation of metanephric mesenchyme with neural tubes induces development of renal tubules, reciprocal interactions between the

metanephric mesenchyme and abundant neural elements might be responsible for renal development. Interestingly, Nanog, whose expression is in large part responsible for maintaining pluripotency in ES cells is located on chromosome 12 and overexpression has been shown to induce primitive ectoderm (of which neuroectoderm is a constituent) (Darr, Mayshar, and Benvenisty 2006).

In a study by Herszfeld et al at the Monash Institute of Medical Research and the Australian Stem Cell Centre from 2006, they examined the expression of CD30 in cell lines from germ cell tumors and in hES cells grown in culture (Herszfeld et al. 2006). CD30 is a member of the TNF receptor superfamily, is present on Reed-Sternberg cells, activated lymphocytes, decidual cells, and is a biomarker of embryonal carcinoma in diagnostic pathology. As expected, RT-PCR products and expression of CD30 was found in the embryonal carcinoma line cells while none was present in the hES cell lines. However, under certain culture conditions (serum free) hES cell lines demonstrated karyotypic abnormalities and overgrew the diploid population. These karyotypic abnormalities included duplications of 1q, trisomy 12, balanced translocation between chromosomes 1 and 6 and a lesion on the long arm of chromosome 1 involving duplications of q2 and an inverted duplication of q3-4 (Interestingly, many of these chromosomal aberrations are present in human germ cell tumors). Teratomas formed by these abnormal cell lines still differentiated into all three tissue types, but had a higher proportion of primitive undifferentiated cells compared to diploid ES cells; a feature seen in teratocarcinomas. In one hES cell line mosaic for trisomy 12, combined indirect immunofluorescence for CD30 and FISH for the alpha-centromeric probe for chromosome 12 showed clear concordance between CD30 expression and the presence of an extra copy of chromosome 12. In addition, CD30 positive cells had lower levels of both spontaneous and induced apoptosis. The authors conclude that the emergence of CD30 expression in aneuploid cell lines may be an adaptive response of the cells to stress permitting survival under suboptimal conditions at the expense of DNA damage.

Prokhorova et al showed defective pluripotency in a hES cell line that had acquired trisomy 20 after 30 passages (Prokhorova et al. 2009). Teratomas (and embryoid bodies in culture) from this line were significantly smaller in size and had a decreased ratio of differentiated to undifferentiated tissues. Tissues present were more primitive and showed ill-defined glandular structures compared to teratomas derived from euploid hES cells. Similarly, Bloch et al demonstrated that 129/Sv mice injected with mES cells with a homozygous deficiency of β -1 integrin developed very small teratomas or did not develop teratomas compared to heterozygous deficient β -1 integrin or wild-type ES cells (Bloch et al. 1997). Other findings in β 1-null teratomas included abnormal distribution of extracellular matrix proteins, partially detached basement membranes, and lack of ES cell derived endothelial cells in teratoma blood vessels. Interestingly, a consistent finding mentioned in a few studies is that blood vessels within teratomas are a chimera between host-derived and ES cell derived endothelial cells. This is in contrast to other tissues present in teratomas that are invariably derived nearly entirely from the exogenous ES cells (Gerecht-Nir et al. 2004; Gerecht-Nir et al. 2003; Gertow et al. 2004; Goldberg Cohen et al. 2006).

2.5 Site of injection

Several anatomical sites of the immunodeficient mouse are used for injection of stem cells for the teratoma assay. These have been chosen for their accessibility to injection, immune privilege status, and ample space to contain a large tumor that still allows the host animal to live and function. The most common sites for injection include intratesticular, subcutaneous,

intramuscular, and kidney capsule. Again, only a few studies have specifically addressed comparison of teratomas from different graft sites. Prokhorova et al injected several hES cells lines at four different sites (subcutaneous, intramuscular, kidney capsule, intratesticular) (Prokhorova et al. 2009). Subcutaneous injections were done with cells suspended in 30% matrigel and without matrigel. The highest efficiency of teratoma formation occurred for kidney capsule injections (10/10, 100%) followed by subcutaneous injections using cells suspended in 30% matrigel (64/68, 94%), followed by intratesticular (60%), subcutaneous without matrigel (33%), and intramuscular (12.5%). It should be noted, however, that all injections were done with approximately 1×10^6 cells although for the kidney capsule injections the number of cells injected was not explicitly defined. The number of injections and efficiencies were difficult to compare due to the wide variation in the number of injections for each site (range 5 [intratesticular] to 68 [subcutaneous with matrigel]). Several cell lines were used and it is unclear if all cell lines were used for all injection sites. This study also did not find any differences in teratoma composition by histological examination, but again tissue types were not quantified. Cooke et al demonstrated that intrahepatic injections of hES cells and EC cells produced rapid tumors 5-8 times larger than those obtained by subcutaneous injections (Cooke, Stojkovic, and Przyborski 2006). Intrahepatic tumors displayed more cystic areas with immature and mature tissue types, higher expression of SSEA3 (embryonic stem cell marker), and lower expression of nestin (intermediate filament present in early tissues with neuronal specification and other tissues) compared to subcutaneous tumors. They postulated that the microanatomy of the liver with open vascular spaces (sinusoidal system) and growth factors (e.g. stem cell factor) produced in the liver contribute to the rapid growth of teratomas. In our experience, we have achieved between 67% and 86% efficiency of teratoma formation when injecting between 5×10^5 and 1×10^6 nhpES cells using the intratesticular location. Some evidence suggests that a post-ischemic environment at least in the central nervous system promotes teratoma formation. Seminatore et al transplanted neuroprogenitors (NPGs) derived from hES cells at various stages of differentiation ranging from early NPGs to differentiated NPGs into rats with either no ischemic lesion or rats with small or large ischemic lesions (Seminatore et al.). The early NPGs had embryoid body-like structures that exhibited characteristics of pluripotent stem cells and expressed markers of pluripotent stem cells. Teratomas formed in the central nervous system of rats of rats injected with NPGs with embryoid-body like structures regardless of the presence or absence of an ischemic lesion. In addition, large ischemic lesions significantly promoted the survival of transplanted early NPGs compared to sham. The authors propose that the post-ischemic environment produces a variety of factors that promote the survival, growth, and differentiation into teratomas from an undifferentiated population of ES cells. In the study by Hentze et al from Singapore, they injected several hES cells lines into kidney capsule, intramuscular, subcutaneous, intraperitoneal, testis, liver, and epididymal fat pad. These authors confirmed the necessity of matrigel for development of teratomas in subcutaneous locations and the teratomas derived from these locations usually lacked large cystic cavities compared to other sites. In their experiments with a limited number of teratomas, intramuscular injections also produced tumors rapidly without significant cyst formation (Hentze et al. 2009).

2.6 Number of cells injected

A few studies have been specifically designed in attempts to answer the question of how many undifferentiated ES cells have to be present in an injection of differentiated cells

destined for therapeutic purposes to produce a teratoma. Cao et al in 2007 demonstrated that a minimum of 500-1000 mouse ES cells containing a double fusion reporter construct of Firefly luciferase enzyme (Fluc) and green fluorescent protein (eGFP) were necessary for teratoma formation after subcutaneous injection (Cao et al. 2007). This same number of cells was also able to generate a faint signal allowing for in vivo bioluminescence live imaging. Fewer cells resulted in no detectable bioluminescence on in vivo imaging and no teratoma formation. For the subcutaneous injections, the number of ES cells injected ranged from 1 to 10,000 increasing by a factor of 10. The ES cells were combined with mouse embryonic fibroblasts and matrigel to equal 100,000 cells for injection into adult nude mice. This same group two years later performed similar experiments using undifferentiated H9 hES cells. In this study at least 100,000 (1×10^5) cells was necessary for teratoma formation after intramyocardial injection into adult severe combined immunodeficient (SCID) mice and at least 10,000 cells were necessary for teratoma formation after intramuscular injection (5/7 tumors).

While not specifically addressed in most studies as a variable, the manner of dissociation of ES cells in preparation for injection or transplantation likely plays a key role in the final number of cells necessary to produce a teratoma. Hentze et al also demonstrated that an increasing number of injected hES cell colonies and single cells produced teratomas more quickly in a linear fashion. In their experiments, hES cell colonies were injected in conjunction with approximately 10^6 human foreskin fibroblasts. Teratoma formation was dependent on the cell line used and its adaptation to trypsin digestion. They found that a collagenase passaged hES cell line did not form teratomas after single cell trypsin digestion (Hentze et al. 2009). It is known that ES cells show increased apoptosis and cell death when dissociated into single cells compared to leaving cells in clusters or clumps. Single cell suspensions may require higher cell numbers to compensate for cell loss.

2.7 Host immunological status

Much less literature has specifically addressed the role of the host animal in teratoma formation. Most investigators use some form of immunodeficient mouse. For our teratoma injections, we have used a severe combined immunodeficient mouse strain that is superimposed on a non-obese diabetic background (NOD-SCID) from Jackson Laboratory. These mice lack T and B cell function within a normal hematopoietic background. Of the studies referenced in this section that specifically address teratoma formation from ES cells, little consistency is present in the strain of immunodeficient mouse used for cell transplantation or injection. This is illustrated in the following table:

Study	Host
(Lee et al. 2009)	SCID mouse
(Cao et al. 2007)	Nude athymic rats
(Kishi et al. 2008)	NOD-SCID and NOD-SCID/ γ^{null} (NOG)
(Gutierrez-Aranda et al.)	NOD-SCID IL2R $\gamma^{-/-}$
(Semiatore et al.)	Sprague-Dawley rats (exogenous immunosuppression with cyclosporine, azathioprine and methylprednisolone)
(Prokhorova et al. 2009)	NOD/MrkBomTac-Prkdc ^{scid} and NOD/LtSz-Prkdc ^{scid}
(Cooke, Stojkovic, and Przyborski 2006)	Adult male nude (nu/nu) mice

Table 1. Immunodeficient hosts used for experimental teratoma formation

The SCID mouse has functional deficits in T and B cells, the NOD/SCID IL2R γ (NOG) mouse lacks functional T, B, and NK cells as well as lacking response to cytokine signals, and the nude mouse lacks T cells. The effect of these immune capabilities on undifferentiated ES cells that are transplanted or injected into them is unknown. As a related aside, work by Koch et al demonstrated that only 20% of undifferentiated ES cells treated with human serum formed teratomas in immunocompromised mice compared to ES cells not treated or treated with heat inactivated serum. They show that the alternate complement pathway is responsible at least in part for dampening the tumorigenic potential of undifferentiated ES cells (Koch, Jordan, and Platt 2006).

In summary, it is clear that both endogenous and exogenous factors contribute to efficiency of teratoma production and composition. It is also clear that more work needs to be done to unravel how specific conditions affect the formation and composition of teratomas. This will include carefully controlled studies and an efficient means to carefully compare the histological composition of the resulting teratomas.

3. Current technology for tracking stem cell fate, monitoring teratoma formation, and delineation of specific tissue types in vivo

3.1 Tracking stem cells and monitoring teratoma formation in vivo

Many investigations producing teratomas have been motivated by finding ways to detect their formation in vivo and molecular modifications to ES cells to prevent their occurrence. This makes obvious sense since their occurrence in the therapeutic setting is undesirable and would not be detectable for weeks to perhaps months after placement or injection of stem cells. The field of molecular imaging has emerged as a means to study organs and track lesions (such as teratomas) both structurally and functionally. In the field of tissue regeneration/engineering it has been used to track the fate of exogenous stem cells or their differentiated progeny. In vivo imaging technologies include magnetic resonance imaging (MRI), computed tomography (CT), positron emission tomography/CT (PET/CT), single photon emission computed tomography (SPECT), charged coupled device (CCD) camera, and ultrasound. Molecular imaging has evolved to allow better understanding of disease using imaging probes tied to a molecular target that can subsequently be followed and imaged in vivo. Two main methods exist for tracking cells or molecular/genetic functions of cells: One, labeling cells directly and two, via use of reporter gene constructs (Kooreman and Wu).

The first labeling modality involves the use of nanoparticles or quantum dots which emit specified wavelengths of light and are photostable. However, delivery to cells can be difficult, nanoparticles tend to aggregate in the cytosol, and can bind to other molecules. Another modality is iron particle imaging where superparamagnetic iron oxide particles (SPIOs) or ultrasmall superparamagnetic iron oxide particles (USPIOs) can be incubated with cells in culture, taken up by cells, and can be followed after injection using MRI. Cells labeled in such a way have been detected in vivo for up to two months. The major drawback is that these particles can be taken up by macrophages and therefore false positive signals can be detected by MRI for extended periods. A third labeling modality is radionuclide imaging. Here cells are given a radioactive compound and imaged using PET or other radioactive detection methods. The main drawback is that cells can be tracked for at most several days and the radionuclide can leak into other cells.

Reporter gene imaging has shown great promise since the stable integration of reporter genes allows progeny of cells to be tracked. These can take the form of an enzyme that

interacts with an exogenous probe to give rise to a signal, a cell surface receptor, a transmembrane protein that mediates intracellular uptake of radioisotopes, or storage protein that concentrates endogenous contrast elements. The expression of reporter genes could be linked to genes of therapeutic or developmental pathway interest and cell progeny tracked by *in vivo* imaging. Depending on the reporter gene construct, detection can be through ultrasensitive CCD cameras (bioluminescence imaging; BLI), PET, and MRI. In the realm of small animal imaging (e.g. immunodeficient mice), several reporter gene constructs have been developed. The double fusion construct of Fluc and eGFP is commonly used. The animal is given a reporter probe, D-luciferin, which in combination with ATP is oxidized to oxyluciferin, AMP, and light at 560 nm. This reaction is catalyzed by the luciferase enzyme (Fluc) (Marques and Esteves da Silva 2009). The emitted low energy photons are detected by ultrasensitive CCD cameras. The green fluorescence protein allows for detection in postmortem histology. Triple fusion reporter constructs have also been designed that use Fluc, a monomeric red fluorescent protein (mRFP) (for cell sorting purposes), and a herpes simplex virus truncated thymidine kinase (HSVtk) for deep tissue PET imaging. The HSVtk phosphorylates its substrate; a fluoridated (^{18}F) hydroxymethyl butyl guanine generating photons detected by PET. Reporter-suicide gene constructs could also theoretically allow detection and elimination of transplanted cells targeting those destined for teratoma formation. In the case of the reporter construct containing HSVtk, administration of ganciclovir to mice for several weeks can result in lack of teratoma formation. Ganciclovir makes use of its specificity for the viral thymidine kinase and is converted to a toxic drug by phosphorylation by the viral thymidine kinase. Thus, cells infected with the virus produce highly-toxic triphosphates that lead to cell death (Kooreman and Wu).

Transgenes specifically designed for MRI have some advantages in that MRI produces three-dimensional images compared to BLI and ultrasensitive CCD camera. A number of MRI reporter genes have been developed including tyrosinase, transferrin receptor, β -galactosidase, and ferritin (Gilad et al. 2007). Ferritin reporter genes have shown no effect on ES cell tumorigenicity or pluripotency and in comparison to SPIOs can be tracked much longer *in vivo* although the signal is orders of magnitude less than SPIOs and thus might be better suited for applications requiring long term monitoring (i.e. gene therapy, tracking cell differentiation or other pathways) (Liu et al. 2009; Cohen et al. 2009). A theoretical drawback of reporter gene constructs is interference of normal cellular function by integration of genes into the nuclear genome.

PET imaging has the advantages of spatial/temporal resolution and high sensitivity. Cao et al were able to visualize teratomas *in vivo* using a radiolabeled RGD (arginine-glycine-aspartate consensus motif present in proteins of the extracellular matrix) that binds to $\alpha_v\beta_3$ integrin during angiogenesis. They demonstrated *in vivo* imaging of established teratomas in athymic mice after injection of ^{64}Cu -DOTA-RGD4 whereas teratomas could not be detected by PET using ^{18}F -FLT (binds to thymidine kinase) or ^{18}F -FDG (glucose analog) (Cao et al. 2009). As noted previously, however, long term tracking of cell fate or development of teratomas is unlikely with this modality due to the transient nature of the radionuclides.

While these imaging modalities and reporter gene constructs are being used to track teratoma formation and eliminate it, these modalities and methodologies could also be used to study the teratoma itself particularly in the areas of development and even tumorigenesis. Linking reporter genes with fluorescent proteins to genes of interest involved in specific pathways of developments or cancer would be extremely revealing.

3.2 Our experience with magnetic resonance microscopy

In our study of teratomas, the main imaging modality used has been high-resolution MRI (Castro et al.). Our goal in using this imaging technique has been to identify multiple tissue types *in vivo* and to correlate MRI intensities with histology. MRI performed at resolutions $<100 \mu\text{m}$ per 2D voxel has been designated Magnetic Resonance Microscopy (MRM), and is used as a tool for virtual histological studies. Advancements in MRM have increased the resolution at which biological samples are imaged to as low as $10\text{-}16 \mu\text{m}$ in 2D images and $20\text{-}50 \mu\text{m}$ in 3D volume acquisitions. Many different "virtual tissue stains" can be imparted on MR histology sections by selecting imaging parameters that take advantage of MR contrast mechanisms. In our studies, teratomas have been imaged after removal from the euthanized mouse host and formalin fixation (see (Castro et al.) for details) using a 30 mm diameter 89 mm vertical-bore 11.7T Bruker AVANCE imaging system (Bruker BioSpin Corporation, Billerica, MA) housed at Carnegie Mellon University.

MRM datasets were roughly correlated with histology to determine what tissue types and structures were reliably identified using MR microscopy. Structures and tissues that could be identified from MRM images with little or no ambiguity were adipose tissue, cyst, cartilage, and epidermal lining. Successful registrations allowed homogeneous tissue patches in histology to be correlated with pixel intensity values in T_2 -weighted images. Tissues with low intensity in MR images, defined as 3-22% of maximum (cyst) intensity, included adipose and necrotic tissues, mature bone/cartilage, and neuroectoderm. Tissues with medium intensity (22-52% of maximum intensity) included neuroectoderm, immature bone/cartilage, skin, muscle, and gastrointestinal. High-intensity tissues (53-100% of maximum intensity) were limited to cystic lining/fluid and gastrointestinal lining. As can be seen, considerable overlap of intensity and tissue types are present (Figure 1). The ability to transform high resolution MRM images to its corresponding histological appearance would be extremely valuable in the study of tissue development in teratomas in a longitudinal fashion. The ability to produce such "virtual histology" requires that MRM images are precisely aligned to their corresponding histological sections from the serially sectioned teratoma. Next the MRM image characteristics (intensity etc.) would have to be correlated with the images characteristics of the tissue in the histological sections. Several hurdles need to be addressed 1) Precise alignment of each histological section with the corresponding MRM images requires alignment in three-dimensions and 2) even with high-resolution scans the voxel dimensions are $42 \mu\text{m} \times 89 \mu\text{m} \times 180$ for MRM and $3.53 \mu\text{m} \times 3.53 \mu\text{m} \times 5 \mu\text{m}$ for a typical histological section; a 1-2 orders of magnitude discrepancy. Single image registration between MRM and histology required tedious and vigilant sample alignment, but did allow for correlation between MR image intensity values and histological tissue types. An additional advantage of MRM is that it can deliver accurate volumetric measurements because true 3D images are obtained, and the sample is unaltered by histological processing.

These higher resolutions did not allow us to uniquely identify any additional tissue types directly from MR images, when compared with clinical imaging. However, MRM may allow direct, non-invasive identification of additional tissue types, because they did reveal texture patterns not visible in low-resolution MRI. Unidentified textures including radial patterns with alternating dark-bright spokes, faint stacks of filamentous parallel lines, dark finger-like projections, faint concentric lines against bright backgrounds, and dark patches with interior textures such as bright speckles or membrane-like lines. Correlating these textures and other regional patterns with the tissues they represent in histology, especially aided

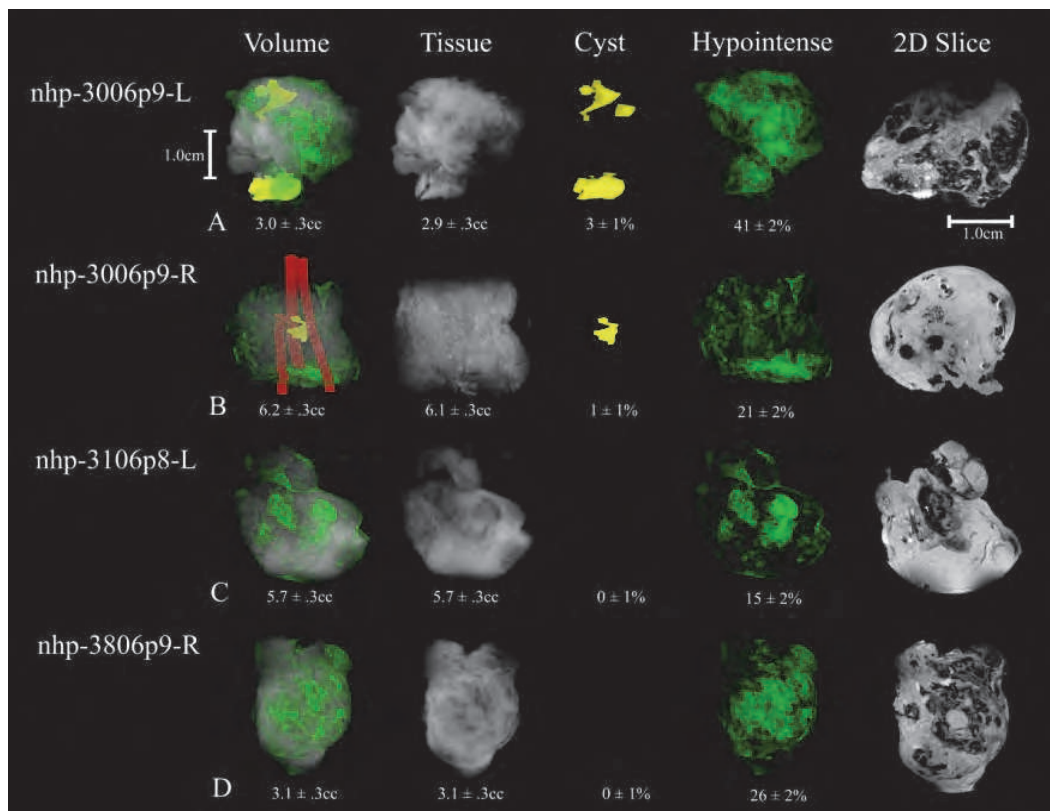


Fig. 1. High-resolution magnetic resonance imaging of teratomas. Teratomas derived from non-human primate ES cells were extracted, fixed and imaged. Hypointense (green; first and fourth columns) and cystic areas (yellow; first and third columns) are segregated and volumes assigned. In the second row, first column, the vertical rods are toothpicks placed into the teratoma at the time of imaging used as fiducial markers for alignment studies. The far right column shows a representative 2D slice.

by computational statistical methods, could identify additional tissue types with varying levels of confidence, directly from MRM of teratomas. Computational textural and shape analysis trained over many teratomas, as well as higher resolutions, may offer improved tissue identification by better resolving and considering these subtle texture variations.

4. Histopathology of teratomas

It seems that whenever we have inspected sections from teratomas under the microscope, we have found something, some structure that wasn't present (or seemingly wasn't present) on previous viewing. This is part of what makes them special and fascinating lesions to study. This emphasizes the numerous and varied developmental programs that are in motion simultaneously as these tumors grow. Few papers have carefully described and/or quantified the numerous specific tissue types present in most teratomas derived from ES cells. The description and quantification of tissue types will be critical if teratomas are to be used as models of development or subtle comparisons of teratomas derived under different experimental conditions are to be unraveled. We have identified the majority of tissue types from the hematoxylin and eosin (H&E) slides without the need for ancillary studies. Briefly, we emphasize two critical aspects of the histopathological analysis that allow for optimal examination of teratomas. One, we underscore the importance of having well-processed and

well-stained H&E sections. This sounds easy, but in reality even though the histological equipment and techniques have been available for decades, the production of excellent slides for accurate tissue assessment, image production and analysis requires great experience within the laboratory. Well-processed tissue includes having the entire teratoma well-fixed (for several days preferably in paraformaldehyde or 10% phosphate buffered formalin for best penetration and antigenic preservation), serially sectioned at 2-3 mm using 130 mm tissue blades, and processed so that it is free of tissue drying and microtome cutting artifacts. This is particularly important since generally speaking, these tumors are large thus generating a tissue section with a large cross-sectional area compared to a small tissue biopsy. We recommend using a regressive H&E stain that provides for excellent nuclear detail and more consistent staining from batch to batch compared to a progressive staining technique (Carson 1997). Two, for detailed critical studies of teratomas, it is essential to have a versatile and experienced pathologist examine all of the tissue sections of the tumor carefully. The pathologist should be comfortable with the microscopic examination of a wide variety of organ systems including perinatal and/or pediatric specimens and experienced in identifying seemingly inconsequential structures within the milieu of tissues present. Perinatal and/or pediatric clinical pathology disciplines tend to have specimens that include fetal organs, organs with developmental arrest/neoplasia, extraembryonic tissues, and clinical teratomas.

Tissue components seen in our experimental teratomas and their germ layer derivation are shown in the table below.

ECTODERM	MESODERM	ENDODERM
Neuroepithelial	Mesenchyme (undefined immature connective tissue)	Pancreas*
Retinal*	Striated muscle	Liver*
Pigmented cells*	Smooth muscle	Respiratory epithelium
Mature neuroglial	Cartilage	Gastrointestinal epithelium
Immature neuroglial	Bone	Thyroid*
Skin	Adipose	Glandular (unspecified) tissue
Squamous epithelium	Kidney*	Yolk sac
	Skin dermis	
	Bone marrow	*-rarely or never seen in our teratomas

Table 2. Representative tissues seen in our teratomas and their germ layer origin

Only one previous study extensively examined in depth a series of teratomas derived from ES cells in the experimental setting. Gertow et al from the Karolinska Institute published their extensive analysis of teratomas derived from one hES cell line in 2004 (Gertow et al. 2004). This is really the first and only one of few papers that performed and documented extensive histopathological, immunohistochemistry, and FISH studies of teratomas derived from hES cell lines. They analyzed 5 teratomas including the application of 30 antibodies to specific antigens associated with various stages of tissue development from all three primordial germ layers. They found several interesting results: One, the vast majority of cells comprising the teratoma was derived from the stem cells with the exception of

endothelial cells comprising feeding vessels and focal areas resembling choroid plexus. Two, they found no cells expressing markers of undifferentiated pluripotent cells. Three, most teratomas had multilineage tissues derived from all three germ layers meaning that different stages of development of the same general tissue type (i.e. mature and immature neuroglial) or different types of the same tissue were present (endochondral, intramembranous bone). Teratomas had extensive and predominant neural development with areas of cartilage, bone, and epithelial development usually within structures reminiscent of bronchi or intestine but derived from both ectoderm and endoderm. A minority of teratomas showed kidney development. Four, more immature areas had higher proliferation rates while mature areas had lower proliferation rates. Five, certain tissue types segregated with one another. Six, differentiation was higher in teratomas grown for longer periods. The latter three points seem to point to some organized pattern following a developmental program. Blum and Benvenisty from the Hebrew University in Jerusalem recently combined three different hES cell lines and produced teratomas that demonstrated equal contributions to tissues from all three lines (Blum and Benvenisty 2007). In addition, analysis of specific structures within the teratomas using laser capture and microsatellite and sex chromosome analysis demonstrated that some structures were polyclonal; that is derived from two cell lines indicating an inductive phenomenon rather than cell autonomous development. Over the last several years, our group has focused on derivation and study of embryonic stem cells. The group has worked with mES cells, hES cells, and has derived several ES lines from non-human primates including several rhesus and baboon lines (Ben-Yehudah et al. ; Castro et al. ; Navara et al. 2007) (Figure 2).

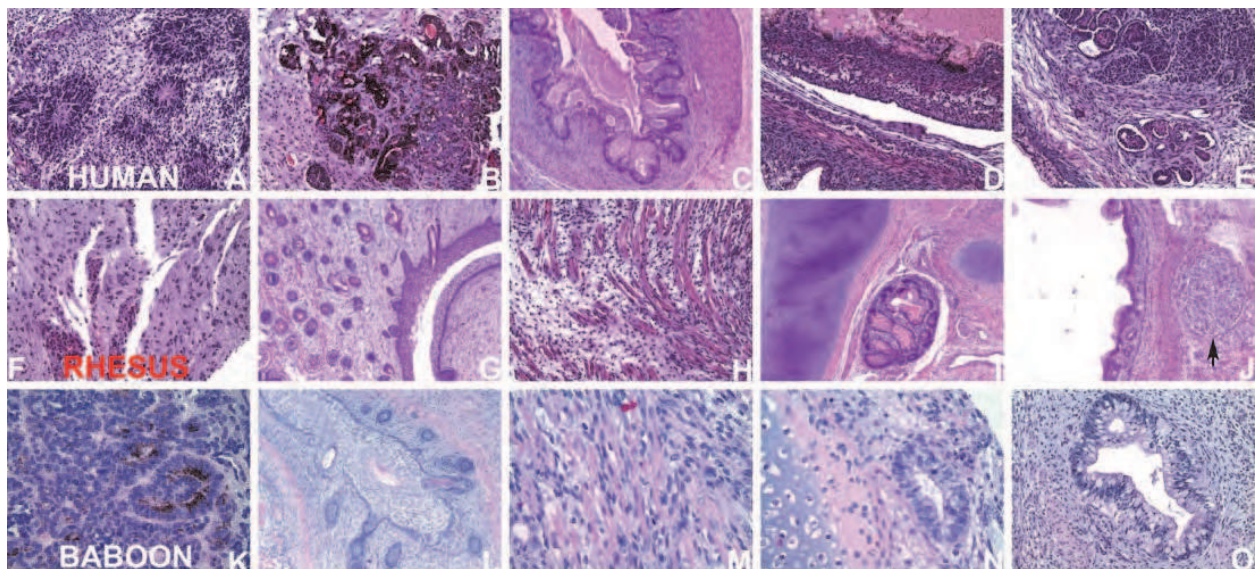


Fig. 2. Representative tissues seen in experimental teratomas derived from human, monkey, and baboon ES cell lines. Human teratomas (A-E): A-neuroepithelium, B-pigmented neural tissue (retinal), C-bowel, D-respiratory or bowel wall, E-primitive kidney. Rhesus teratomas (F-J): F-mature neuroglia, G-skin, H-immature striated muscle, I-immature bronchus, J-bowel wall with ganglion or myenteric plexus (arrow). Baboon teratomas (K-O): K-pigmented neural, L-skin, M-immature skeletal muscle, N-primitive gland adjacent to hyaline cartilage, O-primitive gland surrounded by primitive mesenchyme (H&E stained tissue sections).

More extensive histopathological analysis of teratomas derived from nhpES cells revealed at least 23 different and recognizable tissue entities. (The majority of tissue types present can be seen in Figure 1 of (Castro et al.)). All three germ layers were not only represented (by definition a teratoma) but represented by tissues of the same type at various stages of development and maturation. Neural tissue was represented by primitive neuroepithelium, immature neuroblastic areas, and mature neuroglial tissue resembling brain as well as mature myenteric plexus and ganglia. Skeletal muscle (mesoderm) showed immature myotubes, maturing striated fibrils, and mature striated muscle. Adipose tissue included immature fat containing lipoblasts and areas of mature adipose tissue. Endodermal derivatives included very primitive glandular structures lined by columnar cells with apical nuclei and basal clear cytoplasm (so called “piano key” epithelial cells of yolk sac), less primitive yet still undefined glandular epithelium surrounded by primitive mesenchyme, and well-defined pseudostratified ciliated (respiratory-type) and intestinal epithelium. Immature and mature types of the same tissue could be present in the same teratoma. Only one teratoma had a well-formed tooth organ present.

In summary, teratomas present a dazzling array of tissues at varying stages of development. Tissue recognition, quantification, and delineation of developmental stage are fundamental to their study as models of development, disease, and tumorigenesis.

5. Teratomas in the study of embryonic development

5.1 Quantification of tissues from experimental teratomas

A tumor has been produced in an immunodeficient mouse injected with cells, excised, plopped into formalin, sectioned, a few blocks produced which are sectioned onto slides and examined by the investigator or in some cases a pathologist to determine if tissues from all three germ layers are present. For the most part, if a derived cell line can produce a teratoma and thus prove that the cell line is pluripotent then the teratoma has done its job. The blocks are filed and that is the end. That fact that Muller et al wrote an article calling to standardize not only the reporting of teratoma production but also standardizing the assay speaks to the somewhat nonchalant attitude towards these lesions (Muller et al.). More recent literature has espoused a different look at the teratoma; as a lesion that models development or perhaps failed development since cells are taken out of their normal niche and neighborly contacts (Aleckovic and Simon 2008; Menendez, Bueno, and Wang 2006). Several reasons both practical and theoretical make teratomas an appealing strategy to study development. One, human embryos are inaccessible for studying early development. Embryos at very early stages of development are not available and ethical constraints are enormous. Two, mouse and human development differ significantly such that using mouse models to recapitulate human development is inaccurate. Three, in vitro differentiation of hES cells into embryoid bodies does not reach the complexity of embryonic development. The fact that isolated mouse ES cells can be injected back into a developing blastocyst and continue to contribute to normal embryo formation speaks volumes about how differences in the cellular and environmental milieu can have drastic changes in developmental potential. Some of these cellular and environmental cues in vivo may still be in play even after some degree of cell dissociation in the formation of the experimental teratoma.

We believe that identifying and quantifying the tissue milieu within teratomas both in vivo and ex vivo begin to answer fundamental descriptive questions regarding embryonic development both normal and aberrant. The manual quantification of the myriad of tissues

present in most teratomas is a daunting and time consuming task considering the size of the tissue sections of most teratomas (150 mm²), the number of blocks generated per teratoma (8-12), and the number of tissue types present (>23). A further daunting task is attempting to reconstruct the teratoma in three-dimensional space to visualize the spatial relationships of these tissues to one another. We have done this so far using a semiquantitative approach based on the microscopic assessment of each tissue section. For each section, the amount of each representative tissue belonging to a specific germ layer (i.e. ectoderm, mesoderm, and endoderm) is estimated from each slide of a serially sectioned teratoma using the following scale: 1-[1-20%], 2-[21-40%], 3-[41-60%], 4-[61-80%], and 5-[81-100%]. Estimated percentages are based on how much of the area of the tissue section is represented by that particular tissue. The median of the percentage range is taken for all blocks for each tissue type. For each germ layer, the medians representing the percentage range of the tissue types derived from that germ layer were summed and an overall percentage range assigned to that germ layer. Previously, we have performed semiquantification of tissue types in a limited number of teratomas derived from derived from mES, nhpES, and hES cells with the results in the following table:

	NHP (n=8)	MOUSE (n=3)	HUMAN (n=2)
Incubation (d)	77.8 (13.8)	68.3 (26.3)	70.5 (6.4)
Size (cm)	1.8 (0.3)*	1.9 (0.6)	2.6 (0.1)*
EC (median)	3	5	2
ME (median)	2	1	4
EN (median)	1	1	1

Table 3. Semiquantification of germ layer contributions in experimental teratomas from different species

No differences were seen for incubation days between teratomas derived from nhpES, mES, or hES cells. The human teratomas sampled were significantly larger than either nhp or mES cell derived lesions. Both nhpES and mES cell derived teratomas demonstrated higher median percentage of ectoderm derived tissue present in their teratomas compared to hES derived teratomas. However, hES cell derived teratomas demonstrated higher percentages of mesoderm derived tissues than nhpES or mES cell derived teratomas. No differences were seen for percentage of endoderm derived tissues between nhpES and mES cell derived teratomas. Significant differences at a p-value of 0.02 were seen between the percentage of endoderm derived tissues from nhpES and mES cells compared to hES cell derived teratomas (Ozolek et al. Presented at the 2007 International Society for Stem Cell Research Annual Meeting). While a limited analysis, some trends may exist. One, endoderm seems to be the least prevalent component within teratomas regardless of the species of ES cell from which they are derived. It may be that endodermal derivatives as a whole comprise a small percentage (by weight or volume) of eventual tissues and organs in these species or that the developmental program for certain endodermal derivatives is either not fully established or is aberrant. It is interesting that in our experience with examining numerous teratomas derived experimentally from ES cells, very few contain significant amounts of thyroid, liver,

or pancreas—all of endodermal origin. Two, the amount of ectodermal derivatives seems to inversely correlate with the length of gestation (and by extension length of lifespan) for each species and correlates with the degree of nervous system maturity necessary for survival immediately after birth. The amount of mesodermal derivatives shows just the opposite. One could speculate that the mouse for example has a teleological need to have a more mature central nervous system relative to birth and would need ectodermal derivatives (nervous system, nerves, ganglia, etc.) to be the more abundant and more developed tissue. Our analysis of a large group of teratomas derived from two pedigreed “families” of nhpES cells (same male sperm donor and two different females; 3 ES cell lines in one family and 4 ES cell lines in the other family) demonstrated similar percentage ranges for tissues from each of the germ layers (ectoderm-2, mesoderm-3, and endoderm-1 by the above scoring system) for teratomas of one family compared to the other family (Castro and Ozolek, unpublished data). In all cases, injections of cells were done in both testes usually resulting in two teratomas. Most of these teratoma pairs showed a high concordance of similar tissue types and distributions between each other. Tissue by tissue comparison of teratomas from each of the two families showed no differences in the number of teratomas having a specific tissue with the exception of immature neuroglial tissue which was present in all teratomas from “family 2” and in only half of the teratomas from “family 1” (Castro and Ozolek, unpublished data). These teratomas show a slight predominance of tissue types derived from mesoderm mostly mesenchyme and muscle, followed by ectodermal derivatives (neuroglial and skin) followed by endodermal derivatives represented primarily by respiratory-type or intestinal epithelium (Figure 3). These results have several implications. One, the teratoma doesn’t appear to develop in a single synchronous fashion, in other words; each specific tissue type is not synchronous in its development. Two, some tissue structure (organ formation) is apparent mostly exhibited by bronchial or intestinal development. In fact, in some instances the cross section of an intestinal segment observed in isolation from its surroundings could not be distinguished from that taken from a mature animal or human including the presence of myenteric neuroglial plexuses. Three, very few of these teratomas derived from nhpES cells that we have examined contain significant islands of developed pancreas, thyroid, liver (endoderm) or kidney (mesoderm); all observations also noted by Gertow et al in their teratoma analysis noted above (Gertow et al. 2004).

However, it should be mentioned that Hentze et al also performed similar semiquantification of germ layer percentages in teratomas generated from undifferentiated and differentiated ES cells (cardiomyocytes and beta cells). They found that mesoderm was the predominant tissue type in all teratomas followed by endoderm then ectoderm. Their brief description of the method used for semiquantifying germ layers was similar to ours in that they estimated tissue as a percentage of the tissue area of the slide. According to their methods, they first cut the teratomas into 0.5 gram pieces, then fixed and processed for histology (Hentze et al. 2009). Quantification of tissue from multiple small fragments could prove more challenging and time consuming if one is to examine the whole teratoma. Another source of discrepancy might revolve around how cysts were quantified. The majority of cysts present in a teratoma are going to be endodermal derivatives. In our methods we discounted the lumen as contributing to tissue area since it was an empty space filled with fluid and not actual tissue. The actual tissue element or mucosal lining would actually be a very small percentage of the total tissue present. One could argue the validity of either methodology.

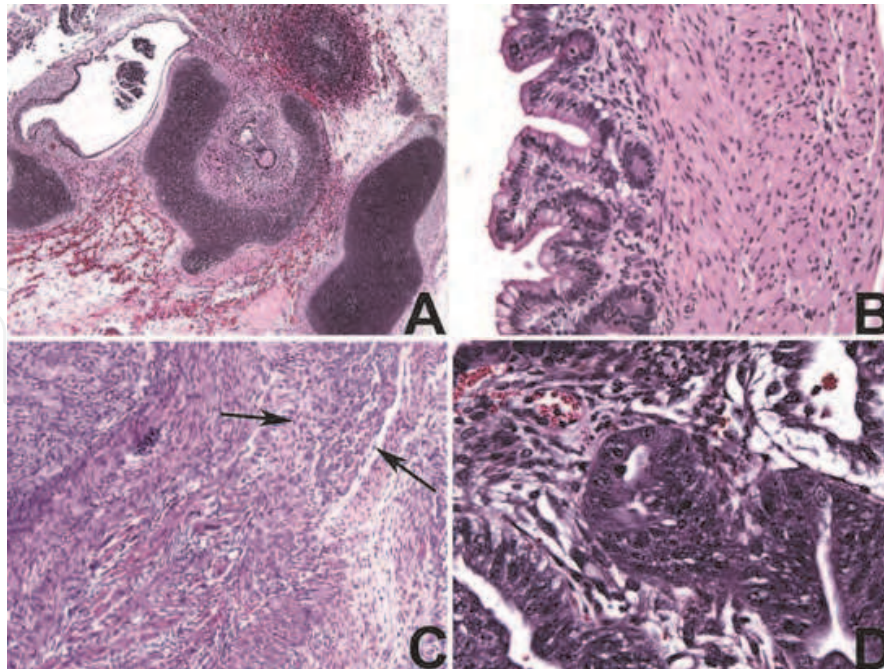


Fig. 3. Common histological themes and interesting observations in experimental teratomas. A-primitive bronchial development (center), B-bowel wall complete with mucosa, lamina propria, and two layers of smooth muscle layered in opposite directions (what would normally be found in mammalian bowel), C-ganglion/myenteric plexus amidst smooth muscle (between arrows), D-primitive glands composed of cells with hyperchromatic nuclei, high nuclear-to-cytoplasmic ratio and basal cytoplasmic clearing (upper right gland) reminiscent of yolk sac (H&E stained tissue sections).

5.2 Automated image analysis for detecting and quantifying tissue in experimental teratomas

As noted earlier, the prospect of manually quantifying tissue types within these teratomas is daunting. Our analysis of the teratomas derived from the nhpES cells took innumerable hours of careful microscopic examination, tabulation, and collation of data and this was only a semiquantitative analysis based on estimated percentages of tissues. Using more rigorous methods would have yielded slightly more accurate data, but would certainly have required two to three times the man hours to accomplish. The ability to accurately and quickly quantify tissues within numerous sections of a teratoma will be critical to identify consistent spatial relationships of tissues with each other, temporal heterogeneity of tissues in three-dimensions and going forward the ability to compare tissue types across multiple teratomas. These authors found only one study that has specifically addressed the issue of quantifying tissues within teratomas using automated imaging/image analysis technologies. Oh and colleagues from Singapore have developed a software platform called TeratomEye that employs three separate algorithms for identifying muscle, gut, and neural tissue with a user-friendly graphical interface (Oh et al. 2009). For muscle, color was used as the main discriminator. For gut, the presence of a lumen was critical in selecting candidates followed by comparison to a set of images of gut and non-gut epithelium. For neural tissue, the same process used for gut tissues was employed. Using these methodologies, they were able to identify muscle accurately 90% of the time with over 90% sensitivity and specificity, gut tissue 87.5% of the time with greater than 80% sensitivity and specificity, and for neural

tissue 47.6%. It is unclear from the images provided in the paper whether all of the epithelium lining structures with lumens was indeed gut epithelium or perhaps early respiratory or other types of epithelium. The images of neural tissue demonstrated only neuroepithelium (with neuroepithelial rosettes) but not maturing or mature neuroglial tissues. A fruitful collaboration with Drs. Kovacevic, Rohde, and several graduate students at the Center for Bioimage Informatics of the Department of Biomedical Engineering at Carnegie Mellon University has resulted in several approaches to the problem of tissue quantification in teratomas. Our work has focused on the two critical components of this analysis: segmentation and identification (classification) of tissues. The ability to automatically identify the boundaries of tissue types and isolate tissues is crucial for both rapid identification and quantification of tissue types. This is of course easier said than done since the teratoma does not always follow our rules, but rather produces tissues closely intermingled with each other often without clear and well-defined boundaries (e.g. striated muscle, mesenchyme, adipose tissue, immature cartilage, immature neural). Segmentation is a critical step in many image analysis pipelines particularly if one is focused on the image analysis of one or several specific regions or areas of interest. In the initial phases of our collaborations, segmentation of tissue types was done manually by the pathologists who by doing so not only identified the region of interest but also provided the ground truth identification for each tissue type. The group at Carnegie Mellon University has made significant strides in developing a segmentation tool that can be applied across a variety of image platforms with minimal customization or "tweaking" necessary by the user based on the type of application (Chen et al. A pixel classification system for segmenting biomedical images using intensity neighborhoods and dimension reduction. in press Proc IEEE Int Symp Biomed Imaging 2011 and Chen et al. A general system for automatic biomedical image segmentation using intensity neighborhoods. in press Int J Biomed Imaging, 2011) (Figure 4).

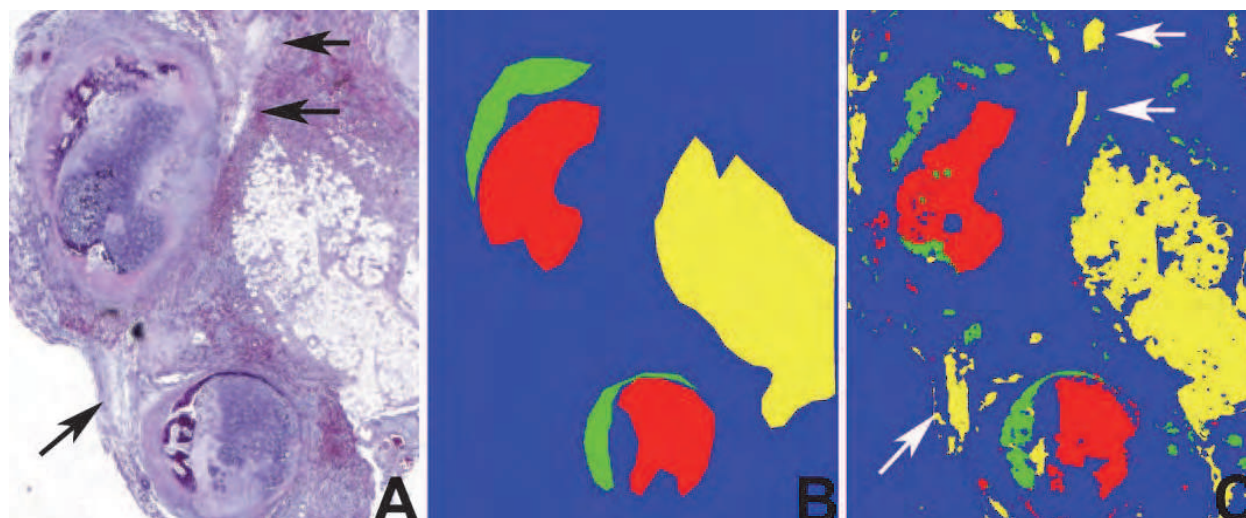


Fig. 4. Automated segmentation tool for distinguishing several common classes of tissues present in teratomas. A-Hematoxylin and eosin stained section of experimental teratoma. Image obtained using a Pathscan II whole slide scanner (Meyer Instruments, Inc. Houston, TX). Cartilage, bone, and adipose tissue (arrows) are readily discernible. B-Ground truth segmented by pathologist: cartilage (red), bone (green), and adipose (yellow). C-Automated segmentation of same image using methods described in text. Note that for adipose tissue in particular, the automated segmentation selected additional areas of adipose not initially segmented by hand (representative regions shown by white arrows).

From an image analysis perspective, tissue segmentation from routinely processed tissue stained with a conventional hematoxylin and eosin stain is challenging due to complex variation in texture, color, shape, and structure of the tissues of interest.

Briefly, this newer approach is achieved within a supervised learning strategy where pixels of a few segmented images are used to train a classifier capable of accurately determining the class (background, tissues of interest) of each pixel in unlabeled images of the same kind. These algorithms use pre-defined window sizes at a given pixel in which pixels inside the window are regarded as neighbors and intensity neighborhood vectors are constructed by reordering the pixels' intensities inside the window into a vector. Using these methods significantly better classification accuracy is achieved across the board for several prototype tissues seen in teratomas compared to other methodologies.

Statistical Measure	Bone	Cartilage	Fat	Background
Our Method	59.70%	73.18%	91.09%	88.93%
Color K-means	29.79%	51.06%	58.73%	55.20%

Table 4. Comparison of quantitative automated segmentation methods for tissue within teratomas

Other approaches aimed at tissue identification have been developed through this collaboration that include use of multiresolution classification, histogram/Earth Mover's Distance, pixel-level classification, and histopathology vocabulary. Multiresolution classifiers have been used since the images (standard H&E) possess many features that are localized in both space and frequency. For these analyses, the image is first converted to grayscale, decomposed into multiresolution subspaces, and features extracted (Haralick texture features and others). A neural network classifier and weighting algorithm are employed to produce a class. Using texture and nuclear features, classification accuracy for several classes of tissues within teratomas approached 88%. The addition of color features improved classification accuracy to over 90% (Bhagavatula et al.). Another approach using the histogram of pixels over the span of a given tissue of interest coupled with a K-nearest neighbor classifier and earth mover's distance produced an average accuracy of 92% for single tissue types (Castro and Bhagavatula. Multiresolution classification of tissue types in teratomas derived from human and non-human embryonic stem cells. Presented at the International Society for Stem Cell Research Annual Meeting 2009). The implementation of a tissue classifier based on the translation of words used to describe histopathological features has proven to be quite robust. In this classification scheme, the pathologist gives each tissue type a verbal description of visual descriptors in order of importance for definitively identifying that particular tissue. For instance in the case of neuroepithelial/neuroectoderm, the verbal descriptors ranked in order of importance to visually identifying it as neuroepithelial/neuroectoderm were the following:

1. Dark blue rim (pseudostratified nuclei) that appears several cells thick
 - Nuclei elongated, oval shape oriented perpendicular to base circumference
2. Round, oval, elliptical macro structure
3. Central lumen (may be light pink and/or white)

This effectively describes the Flexner-Wintersteiner rosettes of primitive neuroepithelium. This was done for 15-20 different tissue types. The engineer then translated those verbal

descriptors into engineering synonyms. The pathologist then checked to see if these translated descriptions were still sufficient to classify the tissues. Based on this process a collection of vocabulary terms was assembled shown below:

Background/Fiber colors	Nuclear shape
Cytoplasm color	Nuclear organization
Clear areas (lumens)	Nuclear orientation
Nuclear color	Macro shape
Nuclear density	Background texture

Table 5. Vocabulary terms used for automated tissue classification

Using this approach an average classification accuracy of 97.75% was achieved over a 10-fold cross-validation for 4 tissue types (Bhagavatula et al Automated identification of tissues for digital pathology in press Transactions on Image Processing, 2011).

5.3 Role of immunohistochemistry in tissue identification and quantification

To this point little has been mentioned about ancillary histological techniques that may be of assistance in the classification and quantification of tissue types within teratomas. This has been for good reason since the goal of our image analysis collaborations has been to develop software platforms that can segment, identify, and quantify tissue from images derived from routinely processed tissue. This means the ability to segment, identify, and classify with high accuracy tissues from the same sections that pathologists' eyes have been using to classify tissues in teratomas and make diagnoses in surgical pathology for decades. The implications are that teratoma analyses can be done without more expensive ancillary histological techniques and increase specificity of tissue identification since as we will see using antibody mediated ancillary methods are not necessarily specific for one tissue type. Having said this, the use of ancillary techniques particularly immunohistochemistry can be very valuable not only for tissue identification and quantification but also key in elucidating developmental pathways. Hundreds of antibodies are available for these purposes with many suitable for formalin-fixed paraffin embedded (FFPE) tissue. The keys to using immunohistochemistry for applications such as tissue identification and quantification are the selection of the antibody, using proper immunohistochemistry techniques, and interpretation of staining. No one antibody stains all tissue derived from each germ layer. Thus, "pan-endodermal" or "pan-ectodermal" antibodies do not exist. In fact, the process is more difficult when considering the teratoma since tissues from all three primordial germ layers are not only present but are present in a developmentally heterogeneous fashion. This means that a single tissue type can be represented as it might appear histologically in early fetal life, late fetal life, and into childhood/adolescence/adulthood. Vimentin for instance is a commonly used antibody in both diagnostic and research arenas and is considered a marker of tissues derived from mesoderm or of mesenchymal origin and is used extensively in diagnostic soft tissue pathology. The antibody reacts to an intermediate filament that is present in most if not all fetal cells in early development and thus non-specific. NeuN is a marker of post-mitotic mature neurons, however, all mature neurons are not stained with NeuN (exceptions include cerebellar Purkinje cells, olfactory bulb mitral cells, and retinal

photoreceptor cells) and immature neurons are not stained. The situation can be even more complicated when considering actin antibodies since 6 isoforms of actin are present, four of which are in muscle (alpha isoforms) and two in non-muscle (beta, gamma isoforms involved with the cytoskeleton and cell mobility). Therefore, a commonly used antibody such as muscle specific actin clone HHF35 will detect the alpha and gamma isoforms but not cells with the beta isoform. Smooth muscle actin, clone 1A4 detects not only smooth muscle but myofibroblasts and myoepithelial cells also. Antibodies are available that will detect actins in smooth, skeletal, and cardiac muscle. Detection of endodermal derivatives particularly yolk sac using IHC can be even more problematic. Antibodies to cytokeratins can be used to detect epithelial linings but are generally not specific for derivatives of endoderm (Dabbs 2006). Alpha-fetoprotein is produced by immature hepatocytes (liver), endoderm, and yolk sac elements but because of its solubility after alcohol-based processing can be variably present upon immunohistochemical staining. Glypican-3 is one of a family of cell surface heparan sulfate proteoglycans that is abundantly expressed in fetal life. In diagnostic pathology, this antigen has been shown to detect yolk sac elements (to the exclusion of embryonal carcinoma, germinoma and most cases of choriocarcinoma and teratoma elements) within human teratomas (Liu et al. ; Zynger et al. 2008). SALL4 also stains most yolk sac elements and is a nuclear stain but it is not as specific as it will also stain seminoma and embryonal carcinoma (Liu et al.). Taking into account the broad developmental spectrum of tissues present in teratomas, available antibodies (that have been tested true in FFPE tissues), and cost-to-benefit ratio (number of consecutive sections that must be done, staining, reagents, antibody cost, interpretation), the following table (Table 6) lists several antibodies that should highlight the majority of tissues present in a teratoma and provide delineation of germ layer derivation. Consecutive sections from each block of a serially sectioned teratoma could be stained with each antibody and reasonably accurate quantification of staining can then be performed using any number of image analysis software packages that can threshold by color/pixel intensity. We say “reasonably accurate” since antibodies only highlight the antigen they were raised against. One should be aware that the vast majority of antigens are typically present in one or at most two compartments of the cell thus an antibody to a particular antigen may only highlight the nucleus (in the case of most transcription factors) leaving the cytoplasm and membrane unstained or may highlight the membrane and not the cytoplasm or nucleus. Image analysis techniques that use threshold intensities will only segment the stained areas and not include the remainder of the cell thereby underestimating the number of pixels that contribute to that specific tissue. This point is illustrated in Figure 5.

Other tissue/developmental stage specific antibodies to consider that are also readily available for FFPE tissues would include myogenin or myoD1 (immature skeletal muscle; marker for rhabdomyosarcoma in diagnostic pathology), thyroid transcription factor (TTF-1; lung/thyroid), CD31 (PECAM-1; endothelial cells), NeuN (Neuronal Nuclei; mature post-mitotic neurons), Sox2 (neuroepithelium within neuroepithelial rosettes, primitive endoderm including yolk sac), S100 (early glia, schwannian stroma of myenteric plexuses, chondrocytes, adipocytes), NCAM (CD56; neural cell adhesion molecule, immature neuroglia), NFP (neurofilament protein, neurons), Ki-67 (proliferation marker for cells in any portion of the cell cycle). Antibodies to Oct4, Nanog, SSEA3, SSEA4, TRA-1-60, and TRA-1-81 are also available for detection of antigens associated with pluripotency, although these antigens are not typically present in teratomas (but can be seen in undifferentiated areas of teratocarcinomas).

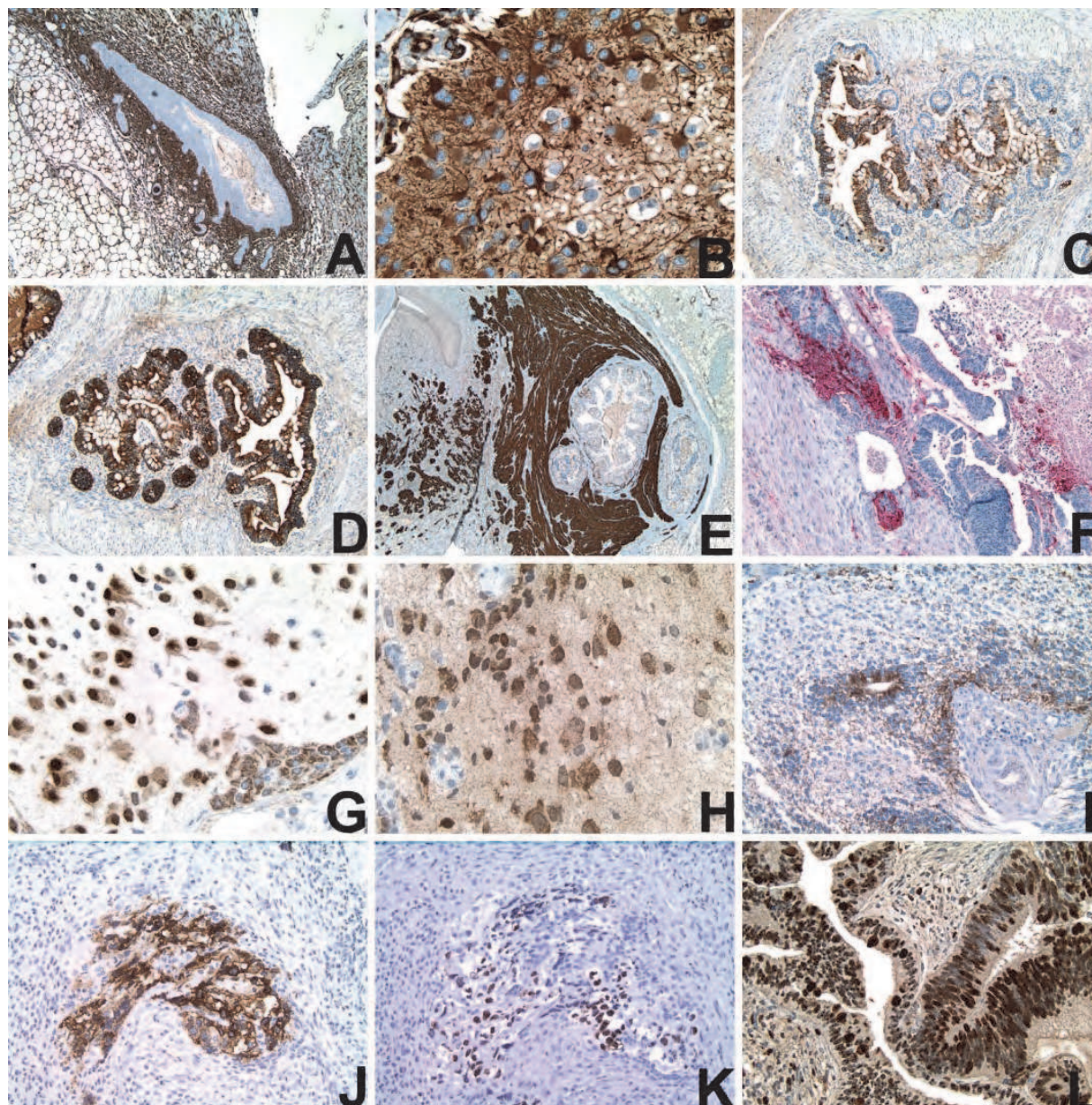


Fig. 5. Sample immunohistochemistry of experimental teratomas. A-Vimentin stained section of skin surrounded by adipose and connective tissue. Vimentin highlights most tissue present but excludes the epithelium (vimentin, 40X), B-Vimentin stained section of neuroglia. In this context vimentin highlights most of the fibrillary background and the cytoplasm of select cells some with cytoplasmic processes (darkly stained cells). These may represent developing astroglia or neural stem cells (vimentin, 200X), C-Cytokeratin 20 stain of epithelial structures. CK 20 is a low molecular weight Type I acidic cytokeratin that is present mainly in colon, pancreas, ovarian surface, and bladder epithelium. In this case the epithelium is focally highlighted (CK20, 100x), D-Cytokeratin 7 stain of same epithelium in (C). CK 7 is present in lung, breast, ovarian epithelium, mesothelium, and endometrium (CK7, 100x). E-All muscle actin stain surrounding mucinous epithelium (AMA, 40X), F-S100 stain of neuroglia highlights cellular and fibrillary staining of area adjacent to more primitive neuroepithelium (S100, red chromogen, 100X), G-NeuN stain of neuroglia showing crisp primarily nuclear staining of neurons (NeuN, 400X), H-PGP 9.5 stain of

neuroglia highlighting neurons and probable neuroblasts (PGP 9.5, 400x), I-glia fibrillary acidic protein (GFAP) stain of human teratoma highlighting some primitive neuroepithelium (GFAP, 200x), J-Glypican-3 stain of yolk sac tumor (taken from child with yolk sac tumor) (glypican-3, 200x), K-SALL4 stain of same focus of tumor seen in (J). Note nuclear staining pattern (SALL4, 200x), L-Ki-67 proliferation marker of neuroepithelium highlights nearly all cells in this focus (Ki-67, nuclear, 200x). Slides of yolk sac tumor in J and K kindly provided by Dr. S. Ranganathan, Children's Hospital of Pittsburgh.

In summary, the preceding sections have only scratched the surface of understanding specific developmental processes. Much of our effort has focused on being able to identify and quantify tissues within these tumors as a means of comparing tumors under experimental conditions. We believe this is a key analysis if teratomas are to be studied to better understand early developmental pathways and the impact of genetic and environmental perturbations on development. Automated image analysis may be crucial in providing the framework for which comparison of tissue composition and quantity can be made between experimental teratomas.

6. Teratomas as models of disease:

Since the isolation of hES cells in 1998, the application of hES cells to human health issues and their potential in this regard has grown exponentially. Directed differentiation of ES cells has produced specific tissue types that may be used for tissue engineering and tissue regeneration. ES cells may be helpful in drug testing, environmental mutagenesis, and toxicity of chemical/physical agents on organ systems and the developing embryo (Ahuja, Vijayalakshmi, and Polasa 2007). They could assist in the identification of molecules that facilitate differentiation into lineage-specific precursors. Applications for developmental biology, disease modeling, and cancer biology are all being investigated (Aleckovic and Simon 2008; Menendez, Bueno, and Wang 2006). In many of these applications, *in vitro* growth and manipulation of the stem cell population is sufficient and desirable. Early but restricted developmental potential is realized with embryoid body formation by hES cells *in vitro*. However, for some applications particularly when more prolonged growth or developmental potential is necessary, *in vitro* models may not be sufficient. Conclusions drawn from murine models of development/aberrant development, cancer, drug toxicity, and other human ailments will always spur some degree of doubt as to their relevance in the human.

The study of tissue development in teratomas could provide a platform for investigating the effects of ingested toxins on the early stages of embryonic development of many different tissue types derived from all three germ layers. This has great significance in understanding the effects of maternal exposure to agents and conditions such as alcohol, tobacco, illicit drugs, starvation, and poor nutrition on the developing fetus. The Barker hypothesis states that fetal and embryonic organ functions can be affected by an altered intrauterine environment and these alterations of organ function determine a setpoint of physiological and metabolic responses that predispose to diseases in adults (Gluckman et al. 2005; Lau and Rogers 2004; Miles, Hofman, and Cutfield 2005). Several epidemiological studies have documented increased deaths due to coronary artery disease and high blood pressure in adults who were born with low birth weights compared to those with birth weights in high percentiles. Intrauterine growth restriction has been linked to impaired glucose tolerance

and diabetes in adulthood, reduced nephron count, hypertension, and thymic dysfunction. Indeed, small-for-gestational age infants show differences in growth and nutritional hormonal status with decreased levels of insulin-like growth factors, insulin-like growth factor binding proteins, leptin and others (Lau and Rogers 2004; Miles, Hofman, and Cutfield 2005). As a model of early human development, analysis of the teratoma derived from hES cells that have been subjected to various agents in culture (nicotine, alcohol) or treatment of the immunodeficient mouse host used for growing the teratoma could prove valuable in studying the molecular events of developing tissues in stressed or adverse intrauterine environments.

We have demonstrated accumulation of Tau protein in neurons within teratomas established from a mutant hES cell line produced by transfection with a mutant presenilin gene (unpublished observations) (Figure 6). This opens the door for teratomas to be used to study specific diseases including neurodegenerative diseases after genetic modification of hES cells.

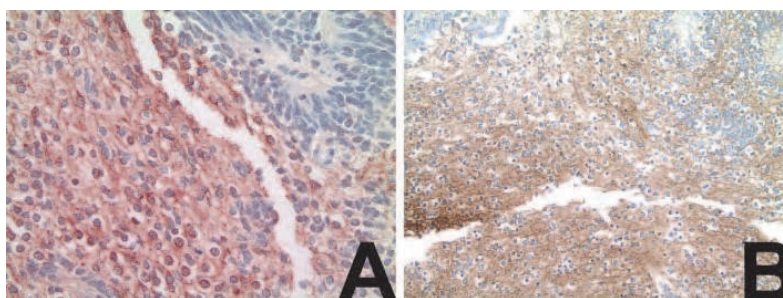


Fig. 6. Tau staining of hES cell line transfected with mutant presenilin gene. A-Staining predominantly in neuroglia adjacent to neuroepithelial rosette (upper right) (Tau, 400x), B- This same area stains with neural cell adhesion molecule signifying its immature state (NCAM, 200x).

Careful analysis of teratomas would begin to unlock the effects on developing tissues resulting from alterations of chromosomal number or specific gene(s). The above mentioned study by Prokhorova et al (Prokhorova et al. 2009) clearly illustrates that alterations of chromosomal number in hES cells can result in dramatic changes in teratoma gross phenotype.

Eiges et al established a hES cell line with full CGG expansion at the FMR1 locus from preimplantation embryos obtained by IVF from a premutation carrier female who had a brother with Fragile X syndrome. Using teratomas derived from this cell line, they were able to show that FMR1 inactivation (and subsequent decrease in Fragile X mental retardation protein (FMRP)) was dependent on differentiation of cells (Eiges et al. 2007). Mensah et al produced a trisomy 21 mES cell line and demonstrated that compared to the wild-type mES cells, teratomas derived from the trisomy 21 cells had significantly decreased amounts of neuroectodermal tissue, decreased mRNA for *Tubb3* (neuron-specific gene) and *gfap* (glial gene), as well as reduced populations of both neuronal and glial cells (Mensah et al. 2007).

7. Teratomas in the study of tumorigenesis

7.1 The unifying theory of teratoma, germ cell tumor, and cancer in humans: More than speculation and theory?

We have already reviewed briefly the close link between ES cells and cancer cells. Mariusz Ratajczak and his group from Louisville have proposed a unifying theory of teratoma, germ

cell tumor, and cancer development in humans. Actually, this idea is not new since several scientists in the 19th and early 20th century have proposed that some tumors result from embryonic rests or germ cells. More recent ideas have the origin of human teratomas and germ cell tumors from displaced visceral yolk sac elements (extraembryonic cell theory), primordial germ cells, and embryonic cells. Recent evidence from imprinting studies shows that primordial germ cells are likely candidates for the cell of origin of pediatric germ cell tumors (Gatcombe et al. 2004; Gonzalez-Crussi, Armed Forces Institute of Pathology (U.S.), and Universities Associated for Research and Education in Pathology. 1982; Gonzalez-Crussi, Winkler, and Mirkin 1978; Kristensen et al. 2008; Oosterhuis and Looijenga 2005; Oosterhuis et al. 2007; Schneider et al. 2001). What is new is that the purported cell to support these past theories may have been found. Ratajczak has isolated from murine bone marrow and organs a population of very small cells (5-6 μm diameter) that have phenotypic features of embryonic stem cells including expression of Nanog and Oct4, high telomerase activity, lack of major histocompatibility antigen-1 expression, abundant euchromatin, and lack antigenic expression of hematopoietic or other stem cells. These cells had probably been excluded by flow cytometry in previous experiments trying to isolate stem cells from tissues due to their small size. These cells termed VSELs or very small embryonic/epiblast-like cells share imprinting patterns similar to those of primordial germ cells (PGCs) with upregulation of growth-repressive maternally imprinted genes (H19, p57, IgfR2) and downregulation of growth promoting paternally imprinted genes (Igf2, Rasgrf1). VSELs remain dormant within tissues and act as a reservoir for tissue specific stem cells that become activated upon tissue injury. The VSELs isolated by Ratajczak regain some of the somatic imprinting profile after about two weeks in culture but also lose expression of Oct4 and thus do not form teratomas in vivo ((Shin et al. 2009) and personal communication, Mariusz Ratajczak, M.D., Ph.D.). They have found certain culture conditions (C2C12 murine myoblast feeders) where VSELs will form embryoid bodies and stain for fetal alkaline phosphatase. They propose that transduction of VSELs with DNA methyltransferase may change imprinting to favor expression of growth promoting and proliferation genes (Ratajczak, Liu et al.) thus promoting experimental teratoma formation. A recent article by Parte et al demonstrates isolation of a similar population of very small embryonic-like pluripotent stem cells from ovarian surface epithelium (Parte et al.).

These authors promote that VSELs are the missing link between the germ line origin of cancer and the current stem cell theory of cancer. Teratomas/teratocarcinomas may form from Oct4+ VSELs that remain dormant in tissues (maintain somatic imprinting and may require additional mutations). Other germ cell tumors may develop from PGCs or VSELs with persistent genomic imprinting and additional mutations. Pediatric small round blue cell tumors (tumors with multipotential phenotypes such as hepatoblastoma, Wilms tumor) could be derived from mutated VSELs in tissues (epigenetic changes; i.e. loss of imprint in H19-Igf2 locus in Beckwith-Wiedemann). Solid tumors may arise perhaps due to chronic inflammation and/or tissue injury that recruit circulating and/or dormant VSELs into damaged tissues with possible heterokaryon formation and the formation of aneuploid cells. (Ratajczak, Shin, and Kucia 2009; Ratajczak, Shin et al. ; Ratajczak et al. 2008; Shin et al. 2009).

7.2 Teratomas as aids in the study of cancer

An interesting proposal by Tzukerman et al uses teratomas not necessarily as a model for tumorigenesis/cancer pathogenesis but as an environmental platform for studying tumor

growth and behavior (Tzukerman et al. 2003). They question the validity of in vitro models to study tumor growth and invasive capability and current in vivo models that use immunocompromised mice. They postulate that murine in vivo models do not mimic what a tumor might do in a human host surrounded by human cells. Their study demonstrated that human ovarian cancer cells injected into teratomas derived from hES cells integrate into and invade surrounding human tissue (within the teratoma) and stimulate neoangiogenesis. They suggest that this type of analysis would be the intermediary step between in vitro and murine in vivo testing of anti-neoplastic agents and human clinical trials. Anti-neoplastic agents could be tested on tumors within a milieu of human cells derived from teratomas.

8. Conclusion

One of the obvious differences between teratomas and a developing human embryo is that we can look at a developing embryo and say (at some point) that the embryo will develop into a human being. We cannot say the same for the teratoma. We really don't know what a teratoma can or will develop into. The dissociation of the blastocyst in reality disarms the entire orderly developmental program forever and when blastocyst cells are collected, reaggregated, and rearranged in a foreign host they simply do not know which end should be up.

Human beings and all life for that matter are amazing in their construction and unconsciously coordinated physiological functions. Form and function are seamlessly woven into a biological tapestry. Teratomas derived from embryonic stem cells are likewise amazing tumors, a visual masterpiece under the microscope; modern or abstract tissue art if you will. Like many examples in the biomedical world, we know who they are but really know very little about what they are. At this point they are more like art in that they have been interpreted individually; "in the eye of the beholder" if you will for the purposes of delineating the presence of three germ layers. Teratomas may be able to help answer many questions. How much of each specific tissue type is present? Do the tissues present recapitulate normal development in three-dimensional space and time? How are the tissues present affected in type and quantity when derived from ES cells that have been manipulated genetically (induced chromosomal aberration, addition/deletion of specific genes) and environmentally (that is, by drugs or toxins, nutrients)? Do all ES cell lines have the equal potential of differentiating successfully into a specific cell type that could be potentially used to treat specific diseases such as Diabetes or Parkinson's disease? How does ES cell phenotype, cell number, site of injection, and host affect tissue formation in teratomas? What are the growth kinetics of teratomas? What influences success rate of teratoma formation? How do teratomas develop? What growth factors influence teratoma growth and tissue development? Do specific developmental programs (tissue or germ layer specific) always show specific developmental potentials? Why does a developmental spectrum (developmentally heterogeneous) of tissues exist within a single teratoma? Why do some "organs" develop more fully than others (i.e. intestine versus kidney)? Why are some tissues hardly ever seen (i.e. liver, thyroid)? Why do teratomas have difficulty forming blood vessels derived entirely from the injected ES cells? Do teratomas differ in their tissue composition between species? At the root of finding the answers to many of these questions will be the ability to carefully, accurately, quantitatively, and quickly delineate tissue types and three-dimensional tissue arrangements both in vivo and ex vivo. Thus techniques for imaging in vivo and ex vivo and image analysis of histological sections

need to be investigated with full vigor and intensity. Targeting specific genes to track expression of known early embryological pathways within teratomas will be the key to unlocking developmental fate and for studying specific human chromosomal and genetic diseases.

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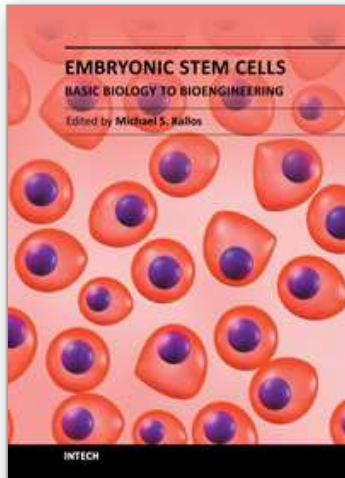
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Embryonic stem cells are one of the key building blocks of the emerging multidisciplinary field of regenerative medicine, and discoveries and new technology related to embryonic stem cells are being made at an ever increasing rate. This book provides a snapshot of some of the research occurring across a wide range of areas related to embryonic stem cells, including new methods, tools and technologies; new understandings about the molecular biology and pluripotency of these cells; as well as new uses for and sources of embryonic stem cells. The book will serve as a valuable resource for engineers, scientists, and clinicians as well as students in a wide range of disciplines.

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