FETAL ALCOHOL EXPOSURE INCREASES SUSCEPTIBILITY TO TUMORIGENESIS IN THE PITUITARY

by

SHAIMA JABBAR

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Dipak K. Sarkar

And approved by

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ABSTRACT OF THE DISSERTATION

Fetal Alcohol Exposure Increases Susceptibility

to Tumorigenesis in the Pituitary

By SHAIMA JABBAR

Dissertation Director:

Dr. Dipak K. Sarkar

Alcohol exposure during gestation increases susceptibility to the development of pituitary tumors in response to estrogen in rat offspring. While the mechanism underlie this effect is not fully understood, serum estradiol (E2) and pituitary aromatase mRNA expression are increased in fetal alcohol exposed (FAE) offspring, which suggests a role for the estrogen axis. To test our hypothesis, pregnant Fischer 344 rats were fed between gestational days 7 and 21 with a liquid diet containing alcohol (AF), pair-fed with isocaloric liquid diet (PF), or fed rat chow (AD). At 60 days of age, animals were ovariectomized and received a subcutaneous estradiol implant. Rats were sacrificed at various times point after estradiol implantation. At the time of sacrifice, pituitaries of these animals were inspected for tumor growth. Estradiol treatment time-dependently increased pituitary weight in AF group as compared to AD and PF groups. After 120 days of

estradiol treatment, inspection of the pituitary revealed that most tumors in the AF group were hemorrhagic and showed expansion to the surrounding tissue. Pituitary tumors from FAE offspring showed strong nuclear p53 and Ki67 expression. Significantly higher mRNA levels of hemorrhage-associated genes and proteins (PTTG, FGF4 and MMP-9) and multipotency genes and proteins (SOX2, Oct4 and CD133) were also observed in pituitary tumor tissues from AF group as compared with PF and AD groups. To test whether FAE enhances the population of cancer stem cells (CSCs) in the pituitary in response to estradiol, pituitaries were collected, and plated in ultra-low plates to promote pituisphere formation. The growing spheres were enzymatically dissociated to permit serial passaging. Assessment of a panel of genes related to multipotency (OCT4, NANOG, KLF4, SOX2, CD133, CD44, nestin and CD34) indicated that mRNA and protein expression of most of these genes was significantly higher in pituitary cells derived from spheres of AF animals as compared to AD. Pituitary cells derived from spheres of AF animals showed higher cell proliferation, migration and colony formation rates as compared to the control group. The pituitary cells derived from AF spheres were able to grow in immunodeficient mice. These data suggest that alcohol feeding enhances pituitary tumor development, and may program the pituitary to express pluripotent and growth promoting molecules under the estrogenic influence to induce aggressive pituitary tumor.

Dedication

I dedicate my humble work to my sweet hearted and loving father, who has always encouraged me to pursue my education.

I dedicate my work to my mother, whose affection, love, encouragement and prayers day and night made me able to achieve this success.

I dedicate my work to all people who are suffering from cancer-associated diseases.

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List of Abbreviations

Pas Pituitary adenomas

IARC International Agency for Research on Cancer

DES synthetic estrogen diethylstilbestrol

ICM inner cell mass

FS folliculostellate

ICI 182,780 estrogen receptor antagonist

ACP craniopharyngioma

AD Ad libitum fed

AF Alcohol fed

BAL Blood alcohol level

CSCs cancer stem cells

E1 Estrone

E2 17β-estradiol

EB 17β-estradiol benzoate

ECM Extracellular matrix

EGFR Epidermal growth factor receptor

EMT epithelial mesenchymal transition

ER Estrogen receptor

ERE Estrogen response element

FASD Fetal alcohol spectrum disorder

GD Gestational day

hESCs human embryonic stem cells

IHC Immunohistochemistry

OVX Ovariectomy

P value probability value

P4 Progesterone

PF Pair fed

PND Post-natal day

IF immunofluorescent staining

ICC immunocytochemistry

NGS next generation sequencing

TSCs tumor progenitor stem cells

PBS phosphate buffer saline

DAPI 4,6-Diamidino-2-phenylindole, dihydrochloride

PFA Paraformaldehyde

IOM Institute of Medicine

ARND alcohol related neurodevelopment disabilities

ARBD alcohol related birth defects

FAEE fatty acid ethyl ester

BEP betaendorphine

POMC Pro-opiomelanocortin (POMC)

ARC arcuate nucleus

DTI diffuse tenser imaging

AAI acute alcohol intoxication

ACTH adrenocorticotropic hormone

PRL prolactin

PRR prolactin receptor

CBTRUS Central Brain Tumor Registry of the United States

GPS germ progenitor stem cells

Chapter 1

Review of the Literature

1- History of fetal alcohol disorder

In 1973, James and Smith observed that alcoholic mothers could give birth to children with morphological defects and developmental delays, which they termed fetal alcohol syndrome (FAS) (Christoffel and Salafsky 1975; Jones and Smith 1973). FAS defects include a set of facial anomalies that consist of short eyelid openings (palpebral fissures), flat midface, thin upper lip, and a flat or smooth groove between nose and upper lip (philtrum). The children diagnosed with FAS also exhibited growth retardation and significant cognitive and/or behavioral problems (Bhuvaneswar, et al. 2007; Jacobson and Jacobson 2002).

In the 1980s scientists defined a new clinical term called fetal alcohol effect (FAE) (Sokol and Clarren 1989). FAE is a general description for children who were previously exposed to alcohol during embryonic development without showing facial abnormalities, growth retardation, or brain damage. However, these children later showed some behavioral abnormalities and difficulties in learning and speaking later in life (Aase, et al. 1995; Clarren and Smith 1978). This made scientists classify them under the category of fetal alcohol effect.

In 1996, the United States' Institute of Medicine (IOM) defined five categories of FAE: FAS with and without confirmed alcohol exposure, partial FAS (pFAS), alcohol-related neurodevelopmental disabilities (ARND) and alcohol-related birth defects (ARBD) (Benz, et al. 2009; Quick 1996). pAE refers to individuals who develop some

facial abnormalities and either growth, central nervous system (CNS) deficits, or a complex pattern of behavioral or cognitive abnormalities. ARND refers to those with CNS deficits or a complex pattern of behavioral or cognitive abnormalities, while ARBD includes individuals with congenital physical abnormalities (Benz et al. 2009).

In 2005 Hoyme et al. (Hoyme, et al. 2005) resolved the ambiguous points of IOM classification by defining Fetal alcohol spectrum disorders (FASDs) based on four digital codes: facial abnormalities, growth retardation, cognitive disabilities and behavioral abnormalities (Benz et al. 2009; Hoyme et al. 2005). Individuals who have one or two of these four digital codes will be defined as having FAS or FAE (Astley 2006). Hence for FASDs the assessment ranking of the four digital points varies between one, which refers to almost complete absence of diagnostic features to four, which refers to a strong presentation of classical diagnostic features. However, because the four digital code system depends mostly on neurodegenerative and cognitive defects, but does not include information related to prenatal alcohol exposure and the offspring's background such as genetic diseases and family history, FASD may be over-diagnosed (Jones, et al. 2006). Therefore, more research, including the identification of biomarkers, is needed to improve FASD diagnosis. For example, alcohol-induced pathology biomarkers like aspartate aminotransferase, and alanine aminotransferase, or some ethanol metabolism derivatives such as fatty acid ethyl ester, ethyl sulfate, and phosphatidyl ethanol, may serve as a potential biomarker to determine prenatal alcohol exposure (Caprara, et al. 2007; Gareri, et al. 2008; Littner and Bearer 2007; Sharma, et al. 2013). In addition, neuroimaging could be used to diagnose brain size and abnormalities in brain shape as they relate to FASD (Lebel, et al. 2008; Moore, et al. 2014; Wozniak, et al. 2006) .

2- Pathology of fetal alcohol exposure

Since Jones and Smith established FAS clinically, researchers started studying the effects of prenatal alcohol exposure on the offspring's immunity and susceptibility to disease. Sharron and her colleagues studied the effects of FAS on the immune system of 13 patients whose age ranged between 12 months to 11 years. Their survey revealed that children with FAS developed lymphocytopenia and neutrophilia. In addition 70% of these patients had respiratory infections such as pneumonia, meningitis and sepsis, which suggested possible abnormalities of phagocytic mechanisms (Johnson, et al. 1981).

There are some clinical evidences in children who exposed to alcohol identified the effect of prenatal alcohol exposure on the neurodevelopmental. One earlier study reviewed the immunity functions of 13 documented cases of FAS, and more recently, one interesting clinical study investigated the effect of prenatal alcohol exposure on the limbic-hypothalamic-pituitary-adrenal axis (HPA). Both studies revealed that prenatal alcohol exposure altered cortisol activity in the offspring (Caputo, et al. 2016; Ouellet-Morin, et al. 2011). Cortisol is a stress hormone that regulates some body functions in response to stress such as immunity and blood glucose (Andrews, et al. 2002; Walker 1996). Another case study was performed to measure insulin like growth factor (IGF), leptin and IGF binding proteins in infants with documented FAS. IGF and leptin were measured as markers for growth development. Interestingly IGF was significantly increased with age in both groups; it was remarkably higher in the alcohol-exposed group compared to control children. Furthermore, leptin levels were significantly lower in the alcohol-exposed group compared to the non-exposed group. The data clearly reveal that

prenatal alcohol exposure can disrupt the normal hormonal functions in offspring (Aros, et al. 2011; Caputo et al. 2016).

Findings from both rodents and primate models strongly support the idea that fetal alcohol exposure significantly alters the HPA axis in the offspring. In addition, adult rat models prenatally exposed to ethanol from gestational day 7 until day 21, which equal the second and third trimesters in humans, have shown over responsiveness to stressors such as lipopolysaccharide (LPS) injection and to drugs including morphine and alcohol (Lee and Rivier 1993; Schneider, et al. 2002; Taylor, et al. 1988; Weinberg 1993; Weinberg, et al. 2008; Zhang, et al. 2005).

Researchers have clearly recorded the harmful effects of chronic alcohol consumption on reproductive function in women. For instance, alcoholic women show a higher rate of menstrual abnormalities as compare to nonalcoholic women (Gabriel, et al. 1998). The menstrual abnormalities include cessation of ovulation, pain, bleeding or irregular menstrual cycles. The alcoholic women who present with menstrual abnormalities show hormonal elevation of estradiol, adrenocorticotropic hormone (ACTH) and prolactin (PRL) (Halmesmaki, et al. 1987). Furthermore, Hankinson et al found that post-menopausal women exposed to 30g of alcohol daily increases levels of RPL and estradiol remarkably as compare to nondrinker women (Hankinson, et al. 1995; Nagata, et al. 2007; Petridou, et al. 1992; Singletary and Gapstur 2001; Smith-Warner, et al. 1998; Stevens and Hilakivi-Clarke 2001; Wuu, et al. 2002).

3- Relationship between alcohol consumption, prolactin levels and incidence of prolactinoma

Prolactinoma is a condition in which a noncancerous tumor (adenoma) of the pituitary gland in the brain overproduces the hormone RPL (Asa 2008). A major effect is decreased levels of sex hormones - estrogen in women and testosterone in men (Charpentier, et al. 1985; Delemer 2009; Gillam, et al. 2006; Klibanski 2010; Melmed, et al. 2002). Endocrinologists in the endocrine and pituitary tumor clinical center at Massachusetts hospital in the USA indicated that 25% of autopsies performed the presence of small pituitary tumors, and mostly were prolactinomas (Charpentier et al. 1985; Delemer 2009; Gillam et al. 2006). Prolactinoma is classified according to the size of the tumor mass; microadenomas are less than 10 mm and macroadenomas 10 mm or more (Charpentier et al. 1985; Delemer 2009; Gillam et al. 2006). The plasma RPL level is related to the tumor size; however, the plasma RPL level can go above the 10000 ng per litter in the case of the pituitary macroadenoma. However, the majority of these tumors are not considered life-threatening, with only 14 out of 100,000 considered clinically significant (Charpentier et al. 1985; Delemer 2009; Gillam et al. 2006; Manuchehri, et al. 2007; Schlechte 2007).

It is now well documented that chronic alcohol drinking increases the level of plasma RPL. An early case study with lactating women was conducted to determine the effect of binge to moderate drinking on oxytocin and RPL levels. The researchers exposed the women to 0.4 g/kg alcohol in orange juice, and RPL secretion was stimulated by the use of breast pumps. Alcohol consumption increased RPL levels significantly compared to women who did not consume alcohol. Furthermore, alcohol consumption decreased

oxytocin level compare with normal women (Mennella and Pepino 2006). An additional clinical case conducted in Germany was employed to measure serum RPL after withdrawal of alcohol in 99 alcoholic male patients compared to 44 normal nonalcoholic subjects. Interestingly the data revealed that RPL levels were elevated during the first 14 days of alcohol withdrawal compare to the control group (Wilhelm, et al. 2011).

The Sarkar lab has studied the effect of binge drinking on RPL levels in cyclic, ovariectomized, and estradiol-17beta-treated ovariectomized Fischer-344 rat models. The animals were divided into three groups: the first group was exposed to 8.7% alcohol in liquid diet (AF), the second group was exposed to an isocaloric liquid diet minus ethanol (PF) and the third group was given a solid diet ad libitum (AD). Interestingly exposure to ethanol increased plasma RPL levels in a time-dependent manner in cyclic, ovariectomized, and estradiol-17beta-treated ovariectomized females. Furthermore alcohol consumption increased wet pituitary weight, prolactin mRNA expression and proliferation labeling index as compare to AD and PF animals (De, et al. 2002). In a study conducted by European scientists, persistent hyperprolactinemia was observed in 16 alcoholic women admitted for 6-week alcoholism treatment at a social hospital (Valimaki, et al. 1990). Furthermore, alcohol-induced hyperprolactinemia has also been demonstrated in 12 men and 9 women with acute alcohol intoxication (AAI).Plasma RPL levels were measured directly when these individuals arrived at the emergency room. Serum RPL levels were 5.8-fold higher in AAI women and 3.5-fold higher in AAI men as compared to control women and men (Frias, et al. 2002). Thus, it appears that chronic alcohol intake in humans results in hyperprolactinemia.

Alcohol also induces hyperprolactinemia in non-human primates and laboratory animals. Studies conducted in female macaque monkeys showed that in some of these monkeys, the RPL levels were elevated after chronic self-administration of alcohol (3.4 g/kg/day) (Mello, et al. 1988). Interestingly, hyperprolactinoma is not only the most common pituitary tumor in humans, but this type of pituitary tumor is commonly found in laboratory animals (Sarkar 2006). The incidence of spontaneous pituitary adenoma was reported in 56% of aged Wistar male and female rats, and in 51% of aged Long-Evans female rats; most of these tumors were prolactinomas (Dipak Sarkar 1983; Sarkar 2010a). The Sarkar laboratory has shown that ethanol increases plasma PRL levels and pituitary weights in cycling and ovariectomized female rats and promotes the E2-induced development of prolactinomas (Chen, et al. 2006; De et al. 2002; De, et al. 1995; Oomizu, et al. 2003; Sarkar and Boyadjieva 2007; Sarkar, et al. 2007). Therefore, these data suggest that ethanol consumption is also a positive risk factor for hyperprolactinemia in animal models. Evidence from animal studies also suggests a detrimental effect of prenatal alcohol on lactotrope physiology. Adult Fischer female rats exposed to prenatal alcohol (35% of the calories derived from alcohol) displayed increased pituitary weights and serum PRL levels in response to estrogen (Gottesfeld, et al. 1992; Sarkar 2010b). Interestingly, it has not been determined if exposure to alcohol prior to birth programs the pituitary to increase the susceptibility to the development of prolactinomas This will be a major focal point of this thesis.

4- The pathogenesis and epidemiology of pituitary tumors:

The pituitary is a bean-shaped organ located in the sella turcica at the base of the skull (May, et al. 2014). This small organ plays critical roles in the maintenance of several

hemostatic functions. Anatomically, the pituitary has two lobes: the posterior lobe and the anterior lobe or adenohypophysis, where most pituitary tumors originate (Al-Brahim and Asa 2006; Asa and Ezzat 2009). The adenohypophysis is composed of six different types of epithelial cells: corticotroph cells, which are responsible for producing (ACTH); somatotroph cells, which are responsible for producing growth hormone GH; lactotroph cells, which are responsible for producing the hormone prolactin (PRL); thyrotroph cells, which are responsible for producing thyroid stimulating hormone; and gonadotroph cells, which are responsible for producing follicle stimulating hormone and luteinizing hormone (Al-Brahim and Asa 2006).

Pituitary tumors are most frequently benign, treatable and non-symptomatic tumors; nevertheless, they can cause a wide range of symptoms, depending on the hormonal mileu and tumor size. The clinical symptoms of excessive hormone production are as follows: growth hormone (GH): acromegaly and gigantism; PRL: amenorrhea, galactorrhea, infertility, reduced libido and impotence; adrenocorticotropic hormone (ACTH): Cushing's disease and hypercorticoidism; and FSH: hyperthyroidism. The effects of intracranial tumor growth include intracranial pressure, visual disturbances, hyper- or hypo-pituitarism and diabetes mellitus (Al-Brahim and Asa 2006; Asa 2008; Asa and Ezzat 2009; Asa and LiVolsi 2008). However, aggressive and invasive adenomas can spread locally to the surrounding tissues such as the bony sphenoid structures and parasellar venous cavernous sinuses (Kaltsas, et al. 2005). These types of pituitary tumors cause many problems due to medical complications and tumor relapse. Pituitary carcinomas are rare; however, the prognosis for this type of tumor is poor (Kaltsas et al. 2005). Whether fetal alcohol exposure increases the incidence of

aggressive and invasive pituitary adenomas or possibly induces pituitary carcinoma is a question this thesis will explore.

According to the Central Brain Tumor Registry of the United States (CBTRUS), the incidence of pituitary tumors is about 2.94 cases per 100,000 persons, and pituitary tumors account for 10-15% of all brain tumors, Glioblastoma represents 45% of malignant tumors, and meningioma represents 54% of non-malignant tumors (Adams, et al. 1993; Minematsu, et al. 2005; Ostrom, et al. 2013; Vankelecom 2012). Interestingly, the data collected by the CBTRUS in the last three years showed that the incidence of pituitary tumors in the US has increased significantly as compared with the previous ten years (Bunin, et al. 1998; Gittleman, et al. 2014). Possible explanations of this increase include improvement in diagnostic methods; however an increased incidence for other reasons cannot be excluded (Bunin et al. 1998; Gittleman et al. 2014). Unfortunately, the CBTRUS report does not provide data regarding the specific types of pituitary tumors, so a precise estimate of the prevalence of each type is not possible (Gittleman et al. 2014) .

5- Characterization of pituitary tumors:

Pituitary tumor classification is a controversial topic; pituitary tumors can be classified based on size, presence or absence of metastasis, type of hormone secreted or morphology (Al-Brahim and Asa 2006; Asa, et al. 1999; Asa and LiVolsi 2008; Asa, et al. 1982). Morphological classification is the most commonly used criterion, and its evaluation depends on histology, immunohistochemistry and electron microscopy. If the classification is made based on the size of the tumor, then the categories are microadenoma (<10 mm), macroadenoma (>10 mm) and giant adenoma (>5 cm) (Asa et

al. 1999; Asa and LiVolsi 2008; Asa et al. 1982). Pituitary tumors can also be classified as intra-pituitary adenomas when the tumors are growing extensively but are not invasive (60-90%), or as invasive adenomas when the pituitary adenomas extend beyond the pituitary capsule and invade the adjacent tissues (10-35%). According to the WHO definition, invasive pituitary adenoma is not regarded as proof of a malignant tumor, i.e. a carcinoma. The WHO considers pituitary invasive adenoma as a carcinoma when the tumor cells invade distant parts of the body, such as the spinal cord and lungs (Al-Brahim and Asa 2006; Asa and Ezzat 2009; Colao, et al. 2010a; Colao and Loche 2010; Colao, et al. 2010b; Kopczak, et al. 2014a; Kopczak, et al. 2014b). According to the WHO classification, pituitary carcinoma can only be diagnosed if distinct cerebrospinal and/or systemic metastasis is documented (Colao et al. 2010a; Colao and Loche 2010; Colao et al. 2010b). Pituitary carcinoma is rare and accounts for less than 1% of pituitary tumors, and these primarily produce RPL and less frequently ACTH (Colao et al. 2010a; Colao and Loche 2010; Colao et al. 2010b). Based on the published clinical cases, pituitary tumors that secrete RPL or ACTH more commonly progress to carcinoma than tumors that do not secrete these hormones, but the molecular mechanisms that signal the transformation from an invasive macroadenoma to malignant disease are poorly understood (Astaf'eva, et al. 2004; Chrisoulidou, et al. 2004). Although some biomarkers such Ki67, P53 and P27 are used to indicate tumor cell proliferation, invasiveness, metastasis and malignant behavior of tumor cells, these markers might be poor predictive markers as their expression levels only change late in the malignant transformation process (Astaf'eva et al. 2004; Chrisoulidou et al. 2004; Yokoyama, et al. 2004). Increased expression of the cell cyclin family genes such as CCNB2, CCND1 and

CDKN1B, and reduced retinoblastoma protein (Rb) have been recorded in pituitary carcinoma and some other types of cancer such as colorectal carcinoma and small cell lung carcinoma (Barakat, et al. 2004; Bombardieri, et al. 2004; Chatzistamou, et al. 2004; Gollini, et al. 2004; Kaltsas, et al. 2004; Komninos, et al. 2004; Limdi and Crampton 2004). Other studies have reported that overexpression of tumor microenvironment genes such as fibroblast growth factor (FGF) and pituitary tumor transforming gene (PTTG) may predict pituitary cancer or pituitary aggressive adenoma (Panguluri and Kakar 2009); similarly, the over expression of these genes is found in breast carcinoma (Bedussi, et al. 2014; Criscitiello, et al. 2015; Koziczak, et al. 2004; Panguluri and Kakar 2009; Solbach, et al. 2004; Watkins, et al. 2010). In addition to the tumor microenvironment genes, high throughput technology has suggested a genetic background of pituitary adenomas (de Lima, et al. 2012). Interestingly, Zhao et al. used a three-stage genome-wide association study (GWAS) to gain insight into the genetic basis of pituitary adenoma; they discovered three new susceptibility loci below the genome-wide significance threshold ($P < 5 \times 10{-}8$) in the combined analysis: 10p12.31,10q21.1 and 13q12.13 (Ye, et al. 2015). Similarly, in the present work we are using RNA sequencing technology to identify novel genes and novel isoforms whose change may be associated with aggressive prolactinomas induced by fetal alcohol exposure.

6- Criteria of invasive pituitary tumors:

Approximately 20 to 25% of pituitary adenomas invade surrounding structures, such as the sphenoid sinus and/or the cavernous sinus (Dai, et al. 2016; Hansen, et al. 2014; Meij, et al. 2002; Scheithauer, et al. 1986; Tampanaru-Sarmesiu, et al. 1996; Thapar, et al. 1996a; Thapar, et al. 1996b). Knowledge of the normal anatomy of the sellar and

parasellar regions can inform our understanding of the anatomical compartments that can be invaded by pituitary tumors. Invasion of the sphenoid sinus can be assessed by different methods: by using imaging techniques (such as CT and/or MRI), or histologically by assessing invasion of the mucosa of the sphenoid sinus. Microscopic identification of invasion to the surrounding tissue, such as dural invasion, is not considered a predictable indicator of aggressiveness (Meij et al. 2002; Sano, et al. 1989; van der Vlugt-Meijer, et al. 2002). According to the Hardy classification system, extension of the tumor to the cavernous sinus is mostly classified as grade 3 or 4 (Knosp, et al. 1993). Giant adenoma is a second type of invasive adenoma, defined by a tumor size of more than 30 mm, or in some studies a cut off of more than 40 mm.

7- Criteria of aggressive pituitary adenomas:

Aggressive pituitary adenomas are classified as either invasive or non-invasive adenoma, microadenomas or macroadenomas. Alternatively they can be characterized as aggressive tumors if the tumor is expansively growing, recurrent, and/or has spread to the cavernous bone or to the skull bone with no response to chemotherapy (Di Ieva, et al. 2014; Rotondo, et al. 2014). According to the WHO, pituitary aggressive adenomas are defined as atypical when they show positive immunoreactivity for MIB-1 Ki67 (labeling index more than 3) and extensive immunostaining for P53 (Onguru, et al. 2004). A prolactinoma is a prolactin-producing adenoma and is the most common type of aggressive pituitary adenomas. Prolactinomas often occur in female patients and are often unresponsive to treatment with dopamine agonists (Delgrange, et al. 1997; Heaney 2011; Komninos et al. 2004; Trouillas, et al. 2000). Resistance to dopamine agonists is the signature criteria of malignant prolactinoma. Malignant prolactinoma is characterized

by relapse within a short time of starting chemotherapy and surgical treatment. A precise histopathological description helps in identifying features of aggressive behavior (Heaney 2011; Komninos et al. 2004). For example, a Crooke cell is one of the important characteristics of malignant and aggressive pituitary adenoma. Crooke cells are non-tumorous cells characterized by hyaline accumulation. Physician Crooke identified the Crooke cell in 1935 in the anterior pituitary gland of Cushings patients (George, et al. 2003; Kovacs, et al. 2005; Saeger, et al. 2007). Crooke cells are corticotropic cells that undergo massive accumulation of glucocorticoid in cytoplasmic vesicles. These cells play a role in producing ACTH and causing Cushing disease. Other work has found that Crooke cells play a role in transforming adenoma tumors to aggressive or cancerous tumors (George et al. 2003; Kovacs et al. 2005). Supporting clinical studies have found features of malignant and aggressive pituitary tumors in more than 60% of patients with Crooke cell tumor. Furthermore, this type of tumor is non-responsive to chemotherapy and recurrence occurs within 1-3 years from the time of surgical removal (Di Ieva, et al. 2012; George et al. 2003; Sav, et al. 2015). In comparison with other types of pituitary adenomas, Crooke cell adenomas are classified as aggressive and malignant pituitary tumors.

Sparsely granulated somatotroph adenomas, densely granulated lactotroph adenomas, acidophil stem cell adenomas, thyrotroph adenomas, plurihormonal adenomas, silent adenomas and null cell adenomas can develop to aggressive pituitary tumors (Asa 2008; Asa and Ezzat 2002; Mete and Asa 2012; Mete, et al. 2012). Infrequently, silent somatotroph adenomas display aggressive behavior and progress to carcinomas (Batisse, et al. 2013; Scheithauer et al. 1986; Vieira Neto, et al. 2013). Both sparsely granulated

somatotroph adenomas and densely granulated lactotroph adenomas are characterized by immunochemical staining for the hormone or by secretory granules that are detected by electronic microscopy or fluorescent microscopy. In sparsely granulated somatotroph adenomas, altered growth hormone (GH) receptor signaling is associated with morphological changes that result in the formation of paranuclear keratin aggresomes (fibrous bodies) (Batisse et al. 2013). Sparsely granulated somatotroph adenomas have imperceptible immunoreactivity for GH, but stain positive with CAM 5.2, an antibody that recognizes cytokeratin-8 and to a lesser extent cytokeratin-7. These tumors are also characterized by a peculiar globular cytoplasmic positivity, strong nuclear staining for Pit-1 and a Ki-67 labelling index ≥3% (Sano, et al. 1991; Thapar et al. 1996a). However, for all pituitary tumor types, the incidence of pituitary adenomas that progress to aggressive or invasive adenomas or of pituitary carcinomas is very small. In this regard compiling further statistical data is of great importance.

8- Biomarkers of aggressive and invasive pituitary adenoma:

While several histological and pathological biomarkers for early diagnosis of invasiveness and aggressiveness have been proposed, no specific marker can decisively predict the pathological prognosis of pituitary neoplasms. Studies of biomarkers use to predict aggressive and invasive pituitary adenoma have been reported (Salehi, et al. 2010a; Salehi, et al. 2010b; Sav, et al. 2012).

Ki-67 (utilizing the MIB-1 antibody) provides a nuclear labelling index and is the most dependable cell proliferation marker for distinguishing proliferating from inactive cells. When the percentage of Ki67 nuclear positive staining detected in a tumor section is

more than 3%, the tumor falls under the aggressive and invasive category. Furthermore, the 3% Ki67 staining cutoff is useful to discriminate invasive from noninvasive adenomas with 97% specificity and 73% sensitivity (Salehi, et al. 2009; Tampanaru-Sarmesiu et al. 1996; Thapar et al. 1996a; Thapar et al. 1996b; Thapar, et al. 1996a). However, there is a lack of standardization of the Ki67 labeling index. In some studies for instance, a Ki67 nuclear value less than 1.5% refers to treatable pituitary adenoma. Other publications have suggested that a Ki67 nuclear value of more than 6% indicates pituitary invasive adenoma and may predict malignancy (Kovacs, et al. 2004; Thodou, et al. 2004). The variance in the use of the Ki67 labeling index to characterize pituitary tumor may have several reasons: tumor heterogeneity, method of preparing tumor sections, choosing a good antibody and the staining protocol, which may lead to the lack of reproducibility of Ki67 labeling index (Salehi et al. 2009, 2010a).

P53 is one of the cellular antigens used as a tumor indicator because it is involved in regulation of cell proliferation. P53 positive immunoreactivity indicates pituitary aggressive and invasive adenoma with a highly predictable relapse and recurrence (Salehi et al. 2009, 2010a). Indeed noninvasive pituitary adenoma presents with no detectable P53 nuclear positive immunostaining, while invasive and aggressive pituitary adenoma presents with more than 10% P53 positive staining and 100% in malignant pituitary adenoma (Thapar et al. 1996a; Thapar et al. 1996b). Interestingly, when high expression of P53 is associated with high Ki67 labeling index, the tumor will classify as pituitary aggressive adenoma and in some cases pituitary carcinoma (George et al. 2003; Salehi et al. 2009, 2010a; Salehi, et al. 2008; Thapar et al. 1996b). More importantly, P53 positive staining associated with Ki67 labeling index, and in association with nuclear labelling of

securin (also known as pituitary tumor transforming gene 1 protein, or PTTG) allowed physicians to classify five of 25 patients presenting with prolactinoma as having invasive adenoma and predicted development malignancy (Wierinckx, et al. 2007). Although the expression of P53 can predict invasive and aggressive adenoma, some studies have not able to detect P53 expression (Salehi et al. 2009, 2010a; Salehi et al. 2008). WHO defines positive staining for P53 as a classification of pituitary invasive adenoma or atypical adenoma. However, its detection can be ambiguous and a definitive method of quantitating p53 expression has yet to be approved (Batista, et al. 2006; Thapar et al. 1996a).

Proteolytic enzymes play an essential role in developing aggressive, invasive tumors because they help to break down the basement membrane, destroy the parenchyma, and connective tissues to allow the tumor cells to migrate and move to the neighbor tissue. Measuring proteolytic enzyme immunoreactivity or or mRNA expression would be useful in characterizing pituitary adenomas. Matrix metalloproteinases (MMPs, especially MMP9 and MMP2) are members of proteinases whose expression correlate with invasive and aggressive tumor and a high radiological grade of tumor (Jaquet, et al. 2003; Trouillas, et al. 2003).

Growth factors have been investigated as reliable biomarkers to categorize pituitary tumors. Epidermal growth factors (EGFs), vascular endothelial growth factors (VEGFs), their receptors (EGFR and VEGFR, respectively) which are expressed by GH-producing adenoma cells and lactotroph-producing adenoma cells, and EGFR and VEGFR levels correlate with aggressiveness of pituitary adenomas (Jaffe and Barkan 1992; LeRiche, et al. 1996). FGFs and their receptors are critical for cell proliferation, regulated

development, stem cell motility, migration of mesenchymal cells and differentiation. Overexpression of FGF4 mRNA expression in pituitary tumor tissue and high immunoreactivity for the FGF family members predict aggressive and invasive pituitary adenoma. Some studies have identified a high level of FGF in blood samples of patients with the most aggressive type of pituitary adenoma (Asa and Ezzat 2005; Mete, et al. 2013; St Bernard, et al. 2005).

Further studies and techniques are needed to identify new reliable biomarkers, which can potentially predict the aggressive and invasive behavior of pituitary tumors.

9- Angiogenesis biomarkers

Angiogenesis is an important process in tumor growth, but no coherent data regarding its role in the development of pituitary adenomas have been established (Lloyd, et al. 2003). Tumor angiogenesis includes two main fields: morphometric analysis of the vessels and characterization of the biological processes that lead to organization and formation of macrovesicles in the tumor tissue. Quantification of the blood macrovesicles in pituitary tumor tissue and comparison to the normal pituitary tissue under normal physiological states may help the pathologist to differentiate between invasive and non-invasive pituitary adenoma as well as help to characterize the pituitary tumor subtypes like secretory adenoma, non-secretory adenoma or nonfunctional adenoma (Jugenburg, et al. 1995; Kovacs, et al. 2001; Lloyd, et al. 2001; Turner, et al. 2000b, c; Turner, et al. 2000d). Macrovesicle density (MVD) measurement is the most reliable method used to quantify macrovesicles in different types of tumors. Several publications have found a positive correlation between MVD and invasive pituitary tumor (Jugenburg et al. 1995;

Lloyd et al. 2003; Turner et al. 2000b). However, meta-analysis and more recent studies have found that MVD is not a reliable indicator of angiogenesis and cannot decisively diagnose pituitary behavior (Di Ieva, et al. 2008). Vascular reshaping could be considered a predictive marker of aggressiveness and relapse and chemotherapy resistance, but currently there is no such standardized and accepted method available. Computer methods to measure geometric diameter of MVD are the most reliable methods to assess pituitary tumor angiogenesis. However, this method is limited to research work, despite attempts to use it in the clinic (Di Ieva et al. 2008; Di Ieva, et al. 2013). Understanding the molecular biology of angiogenesis is challenging in brain and pituitary organs. Next generation sequencing, genetic, and epigenetic studies need to be employed to understand the mechanisms that lead to angiogenesis and unmask their role in the development of aggressive and invasive pituitary adenoma (Carmeliet and Jain 2011; Liu, et al. 2003; Zachary 2003a, b).

Evidence suggests that VEGF plays an important role in promoting invasive pituitary tumors by an undefined mechanism. Some studies have found that using tyrosine kinase inhibitors that target the VEGF receptor, such as sorafenib or sunitinib could be useful to treat patients with a pituitary tumor (Barroso-Sousa, et al. 2014; Barroso-Sousa, et al. 2013a; Barroso-Sousa, et al. 2013b; Sanchez-Tejada, et al. 2013). However, more studies need to be done to uncover the role of VEGF in the development and progression of pituitary adenomas (Lepore, et al. 2006; Lloyd et al. 2003) . Therefore, VEGF cannot be use as a conclusive marker of aggressive and invasive adenoma.

Angiogenesis growth factors have been widely studied in other types of cancer such as glioma. For example, studies have found hypoxia-inducible factors (HIFs) play critical

roles in the poor prognoses of glioblastoma. In pituitary tumors studies have reported that amplification of HIFs in invasive pituitary adenoma while some other studies have reported that HIF expression is elevated in pituitary carcinoma (Kaur, et al. 2005; Lepore et al. 2006; Lloyd et al. 2003; Sav et al. 2012).

Endocan (also known as ESM-1) is a proteoglycan secreted by endothelial cells (Maurage, et al. 2009; Scherpereel, et al. 2003) and has been described as a marker of neoangiogenesis. Interesting studies have found a strong correlation between positive immunostaining for endocan and pituitary tumor recurrence and chemotherapy resistance (Cornelius, et al. 2012). Furthermore, correlation of endocan expression with some other proliferation markers including P53 and Ki67, make endocan a promising biomarker of aggressive and invasive pituitary tumors (Cornelius et al. 2012).

PTTG is correlated with angiogenesis (Sav et al. 2015; Sav et al. 2012) and, in pituitary tumors, PTTG mRNA levels represent strong positive correlation with angiogenesis factors including VEGF and VEGFR mRNA (McCabe, et al. 2002). Further, both knocking down and overregulating of PTTG promotes chromosomal instability and aneuploidy, and deletion of PTTG abolishes tumor growth by enhancing P53 and P21 dependent apoptosis pathways (Chesnokova and Melmed 2009, 2010; Chesnokova, et al. 2010).

10- The molecular mechanism of pituitary tumorigenesis

Several studies have attempted to define the genetic keys of pituitary tumorigenesis (Lloyd 2004). Studies have demonstrated that mutations in oncogene RAS strongly correlate with invasive and malignant carcinoma (Karga, et al. 1992; Pei, et al. 1994).

Next generation sequencing including microarray, RNA sequencing and comparative genomic hybridization has found genes whose expression is impaired in invasive pituitary tumors and in somatotroph pituitary adenomas (Wierinckx et al. 2007). Furthermore, variants in the gene encoding GHRH are predictive of tumor aggressiveness (Thapar, et al. 1997a; Thapar, et al. 1997b). However, our understanding of pituitary tumorigenesis, invasiveness and pituitary carcinoma needs more investigation.

Some of these approaches to study molecular mechanism have determined that mutations in angiogenic growth factors, a Ki67 labeling index > 10% and P53 positivity >5% and allelic loss of chromosome 11 may be indicators of pituitary carcinoma (Zemmoura, et al. 2013). However, it must be stressed that pituitary carcinomas often lack these changes, and aggressive pituitary adenomas might express similar markers as carcinomas (Rickert, et al. 2001). In general there is a wide similarity in appearance between the different types of pituitary tumors.

Multiple endocrine neoplasia (MEN) type I (MEN1) is an autosomal dominant disorder characterized by endocrine tumors of the pituitary gland, parathyroid gland, endocrine–gastrointestinal tract and pancreas. MEN1 is a tumor suppressor gene present on chromosome 11q12, and translates to menin, a known nuclear protein. Menin protein plays a role in regulation many cellular functions like transcriptions, genomic stability, cells proliferation and division. Patients with MEN1 mutation usually have a family history of the disorder, and MEN1 mutations can be identified in 70–95% of patients. In patients with MEN1, pituitary tumors are usually diagnosed at an earlier age, have a higher degree of aggressiveness and invasiveness, are more often resistant to treatment,

and have higher rates of tumor recurrence than sporadic pituitary adenomas (Syro, et al. 2012; Trouillas, et al. 2008).

Bone morphogenetic protein (BMP) is one of the secreted transforming-growth factor beta superfamily that play a crucial role in anterior pituitary cell commitment. There are at least two members of this family, BMP2 and BMP4 (Takuma, et al. 1998; Zhao 2003). BMP4 plays a critical role in early pituitary development in which it expresses from ventral diencephalon at 9.5-day post cotium as the infundibulum makes direct contact with Rathke's pouch. Although the BMPs family has more than 20 members, BMP2 and BMP4 are the most important factors that play essential roles in pituitary tumor development (Kahata, et al. 2004; Kawabata, et al. 1998). Recently, Marcelo Páez-Pereda and his colleagues have shown that noggin and BMP2 mRNA expression are downregulated in $D2^{-/-}$ mice, which is very similar to other prolactinoma models including estradiol-induced prolactinoma in the rat model and human pituitary adenoma (Fiorentini, et al. 2002). This evidence is consistent with in vivo and in vitro studies, which reveal reduced pituitary tumor growth in nude mice by overexpression of noggin or smad4dn, one of the TGFβ families (Delidow, et al. 1991; Peluso, et al. 1991; Ramsdell 1991). TGFβ plays a significant role in inhibition of the oncogenic expression of c-Myc protein (Chi, et al. 2015; Mullen and Wrana 2017). However, BMP-4 over growth signaling in the control of PRL-secreting cells cancels the inhibitory effect of TGFβ. Similarly, in vivo experiments using GH3–Smad4dn cells (which do not respond to either BMP-4 or TGFβ) fail to induce tumors in nude mice. Hence, noggin as a BMP-4 blocker, and Smad4 dn blocked tumor growth in vivo. Hence, the results obtained with

Smad4 dn clones strongly suggest that BMP-4 stimulates prolactinoma growth, whereas TGFβ inhibits it.

Wnt proteins are autocrine secretory molecules playing a significant role in cell proliferation, differentiation and migration. Wnt1, initially called integration 1 (Int-1), was described in virally induced murine breast cancers (Clevers and Nusse 2012; Guardavaccaro and Clevers 2012; Schepers and Clevers 2012). There are three principle pathways of Wnt proteins: canonical (β-catenin), non-canonical also known as the 'calcium' pathway, and the 'planar cell polarity' pathway. The canonical β-catenin pathway triggers when Wnt interacts with its membrane receptor frizzled (Fz) to deck other co-receptor includes lipid-related peptide 5/6 (LRP5/6) to form heterodimeric complex receptor for Wnt proteins. Then Wnt/βcatenin signaling transduce to activate nuclear transcription factors including T-cell factor (TCF) and lymphocyte enhancing factor (LEF) to utilize multiple cell activities (Clevers and Nusse 2012; Schepers and Clevers 2012; Yi, et al. 2011). Wnt/β-catenin plays an essential role in the normal development and tumor development in different tissues and organs including endocrine tissues (Poutanen 2006). Although the role of Wnt signaling in breast cancer development has been addressed by many studies (Benhaj, et al. 2006), the exact mechanism of Wnt signaling in subtypes of pituitary tumors is not fully understood. Interesting clinical study on 43 pituitary adenoma specimens were obtained from patients exposed to surgical operation and normal control pituitary tissues were collected at autopsy. The study found that Wnt/β-catenin mRNA and protein expression strongly correlated to the invasive grade of pituitary adenomas (Li, et al. 2014). Furth more, Wnt4 knock out mice show hypoplasia of the anterior lobe of the pituitary gland with a

significant reduction in the GH, PRL and ACTH cells population as well as reducing αGSU, which is alpha glycoprotein hormone subunit common to LH, FSH and TSH (Potok, et al. 2008; Rosenfeld, et al. 2000; Treier, et al. 1998). Other studies have shown that overexpression of Wnt4 in the pituitary adenoma due to downregulation of the Wnt inhibitory factors WIF1 consequently develop to pituitary invasive or aggressive adenoma (Elston, et al. 2008). Hence, Wnt signaling may play a role in pituitary tumor development, and in particular GH producing adenoma, PRL producing adenoma, and TSH producing adenoma.

Sox2 is a member of high mobility group (HMG) box DNA-binding domain called Sox family that contain from 79 amino acids and sharing more than 50% homology on the sex-determining gene SRY. SRY is sex determination Y chromosome discovered in 1990, which is solely responsible for sex determination. SRY has conservative DNA binding domain that geometrically designed to bind to the minor groove of DNA, which gives SRY the biological effect in sex determination. Mutation in the binding protein leads male in to female sex reversal (Gubbay, et al. 1990; Keramari, et al. 2010; Koopman, et al. 1991; Pontiggia, et al. 1994; Sinclair, et al. 1990). Emerging evidence indicates that Sox2 protein play appreciated role in Wnt-target gene expression. Other evidence implicates that Sox2 gene expression is regulated by Wnts, and together these Sox-Wnt interactions appear to play spatial temporal role in activation of canonical Wnt signaling in embryonic and cancer development (Castinetti, et al. 2011; Gaston-Massuet, et al. 2011; Kormish, et al. 2010; Zhang, et al. 2008; Zhang, et al. 2009). Gaston and colleagues have done an interesting experiment on $Hesx1^{Cre/+}$; $Ctnnb1^{+/lox(ex3)}$ mice line drive activation form of β-d-galactosidase expression in the periluminal progenitors cells

at 9.5–10.5 dpc and in committed differentiated adult cells (Andoniadou, et al. 2007; Gaston-Massuet et al. 2011). Activation of Wnt/β-catenin signaling in progenitor stem cells, but not in the adult differentiated pituitary cells, led to development of a pituitary tumor resembling the pediatric form of human craniopharyngioma (Alatzoglou, et al. 2011). Furthermore, the overexpression of mutant β-catenin in Sox2-Cre lines activate pituitary tumorigenesis. Like the human craniopharyngioma (adamantinomatous craniopharyngioma, ACP), the main target is the nuclear accumulation of mutant β-catenin, which leads to tumor development (Alatzoglou et al. 2011).

11- Pituitary gland and stem cells

The pituitary gland has a low rate cell turnover (Levy 2002) and is composed of six hormonal-responsive cell populations that are regulated by certain physiological stages such as puberty, sexuality and lactation (Nolan, et al. 1998). Pituitary progenitor/stem cells play important roles in the regulation of hormone synthesis and secretion under normal physiological demands (Carbajo-Perez and Watanabe 1990; Rizzoti 2010; Taniguchi, et al. 2002). The idea of pituitary stem cells has been widely supported by Yutaka and his colleagues when they double labeled the anterior pituitary mitotic cells with hormone specific antibodies. They found less than10 percent of these cells differentiated into different hormone secretory cells while the other portion remained to undifferentiated. In addition, 30 to 40 percent of the differentiated cells were identified as GH and PRL-producing cells, indicating the potential activity of pituitary stem cells towards GH and PRL hormonal producing cells (Candolfi, et al. 2002; Taniguchi et al. 2002; Tierney and Robinson 2002). Later, Chen *et al* has supported the idea of pituitary stem cells by staining the anterior pituitary lobes of adult mice for verapamil-sensitive

Hoechst dye, which is specific for stem/progenitor cells. More importantly flow cytometry and immunofluorescent staining reveals that the Hoechst dye labeled a cell population that expresses stem cell specific antigen (Sca1). Interestingly, by studying these cells, they found that Notch, Wnt, and sonic hedgehog signaling pathways, which are known to be involved in stem cell renewal and fate decision, are highly activated, suggesting a potential molecular mechanism (Chen, et al. 2005).

Endocrinologists have provided many studies that support the hypothesis of pituitary stem cells. Yoshimura et al did the first such study by transplanting purified chromophobes or amphophils cell at the intrahypothalamic sites in hypophysectomized rats. Chromophobe cells refer to the type of the anterior and intermediate pituitary epithelial cells that their cytoplasm does not stain readily. After 24hrs,the Chromophobe pellets started increasing their mitotic activity, then differentiated into acidophilic and basophilic cells, which suggested that chromophobes pellet worked as stem cells pool (Yoshimura, et al. 1969). About ten years ago, Chen and his colleagues successfully isolated progenitor stem cells from the anterior pituitary gland of Green fluorescent protein (GFP) transgenic mice. They labeled the anterior pituitary lobe suspensions with R-PE-labeled anti-Sca1, which is a general stem cell antigen marker to sort the stem cells by fluorescence activated cell sorting (FACS) technique. Interestingly, Sca1 positive cells shown expression of most of the common progenitor transcription factors including Sox2, Oct4, HHS, Nanog, nestin, prominin-1, and Bmi-1, and members of the Notch (Notch1 and Hes1), Wnt, and Shh (Ptch1) (Chen et al. 2005; Chenn 2008). Eventually, simultaneous studies reported convincing evidence that support the existence of facultative stem cells in the pituitary gland (Chen, et al. 2009; Fauquier, et al. 2008;

Garcia-Lavandeira, et al. 2009; Gleiberman, et al. 2008). Taken all together, the data suggest that the stem/progenitor positive cells are indeed present on the marginal zone of the Rathkes pouch remnant, which in the past repeatedly identified as a pituitary stem cell niche (Carbajo-Perez and Watanabe 1990; Correr and Motta 1981; Wilson 1986; Yoshimura, et al. 1977).

While it is clear that cells with stem cell characteristics exist in the pituitary gland, more studies are need to identify their functionality and their contribution to organogenesis and disease development including cancer progression and repair mechanisms.

12- Role of stem cells in pituitary tumor development

Considerable evidence supports the existence of cancer stem cells (CSCs) in pituitary adenomas isolated from mice and humans (Chen, et al. 2014; Hosoyama, et al. 2010; Lloyd, et al. 2013; Mertens, et al. 2015; Orciani, et al. 2015; Tunici and Yu 2009; van Rijn, et al. 2013; Xu, et al. 2009; Yunoue, et al. 2011). Identifiers of CSCs include some or all the following criteria: self-renewal, multipotent proliferation ability, resistance to chemotherapy, ability to proliferate to progenitor stem cells (PSCs) and ability to constitute tumors in immunodeficient experimental animals. Recent reports have studied stemness features of ACP pituitary tumors in children. The researchers found positive immunoreactivity for markers of adult pituitary stem cells (Sox2, Klf4, Nanog, Oct4 and β-catenin) in human pituitary tumor samples (Garcia-Lavandeira, et al. 2012). Of note, in the ACP mouse model, investigators have not found Sox9 expression in β-catenin cell

clusters but in the cells, surrounding the clusterSox2 expression is clearly upregulated (Andoniadou, et al. 2012; Andoniadou, et al. 2011; Gaston-Massuet et al. 2011).

It has been found that the expression of a nuclear form of βcatenin in Rathke's pouch precursors in the mouse (*Hesx1Cre/+; Ctnnb1lox(ex3)/+* mouse model) is enough to develop pituitary tumors similar to human ACP (Gaston-Massuet et al. 2011). Rathke's pouch marks the beginning of the anterior pituitary and contains a group of undifferentiated cells that are characterized by self-renewal and the ability to differentiate to all the other hormone-producing cells of the anterior pituitary gland. ACP tumors are characterized by nuclear βcatenin accumulation, which leads to activate Wnt signaling pathways. Furthermore, microarray and gene clustering analysis have reported similar structures in human and mouse ACP (Andoniadou et al. 2012). Some studies have identified CSC transcription factors in human ACPs, but no functional characterization has been determined (Garcia-Lavandeira et al. 2012; Holsken, et al. 2014). Mouse ACP tumors, however, contain a cluster of cells with the ability to self-renew. These cells are capable of forming progenitor cells and differentiating into hormone-producing cells *in vitro* (Gaston-Massuet et al. 2011). As compared to the normal mouse pituitary gland, mouse ACPs are composed of higher numbers of cluster cells when cultured in stem cell medium, suggesting the existence of stem cells compartment in this type of pituitary tumors. In addition, stem cells generated from mouse ACP tumors express stemness markers such as Nestin, Sox2 and Oct4, and express low level of differentiated markers like Pit1 and S100. Furthermore, the cells are characterized by higher proliferation rates as compared to normal pituitary stem cells generated from the normal mouse pituitary gland (Gaston-Massuet et al. 2011). In conclusion, human and mouse model studies have

supported the role of CSCs in pituitary tumorigenesis. More experiments need to be done to enhance our understanding about the role of normal stem cells in the pituitary gland and improve our ability to delineate the possible role of stem cells in pituitary tumor formation. This may lead to the development of new possible biomarkers and effective diagnoses.

13- Pituitary carcinomas

Pituitary carcinomas are defined by the presence of metastases. Whether spread of the tumor to the brain and sella tissue is also a definition of carcinoma is a matter of controversy (Gaffey, et al. 2002; Trilck, et al. 2005). Pituitary carcinoma is very rare; however, most of the defined cases to date are either PRL-producing tumors or ACTH-producing tumors (Gaffey et al. 2002). Interestingly, most pituitary carcinomas originate from invasive relapsing adenomas, or from tumors that had undergone surgical removal or radiation therapy, which may stimulate the tumor cells to invade the surrounding connective tissue and migrate through the blood vessels (McCutcheon, et al. 2000; Quevedo, et al. 2000; Salpietro, et al. 2000). Most of the published reports of pituitary carcinomas show a higher index of Ki-67 and p53 protein and a lower expression of p27 (Roncaroli, et al. 2003; Weber, et al. 2003) in the primary tumor and its metastases. Ras mutations can be found in PRL-secreting carcinomas (Cai, et al. 1994). However, more studies are needed to differentiate these pituitary carcinoma which may exhibit similar features.

Chapter 2

Fetal alcohol exposure increases susceptibility to tumorigenesis in the pituitary gland in ovariectomized rats exposed to estrogen: in vivo evidence

Abstract

It is widely accepted that exposure to adverse environmental conditions and lifestyle choices during pregnancy can result in fetal programming that underlies disease susceptibility in adulthood. Fetal alcohol-exposed offspring display many behavioral and physiological abnormalities including neuroendocrine-immune functions, which often persist into their adult life. Since the neuroendocrine-immune system is critically involved in the regulation of tumor surveillance, we sought to determine whether fetal alcohol exposure increases the susceptibility to estrogen-induced pituitary prolactin-secreting tumors (prolactinomas) commonly occurring pituitary tumor in humans. Pregnant Fischer 344 rats were fed between gestational days 7 and 21 with a liquid diet containing alcohol (AF), pair-fed with isocaloric liquid diet (PF), or fed ad libitum with rat chow (AD). At 90 days of age, some of the female offspring were sacrificed on the day of estrous and used for determination of pituitary functions. The remaining animals were ovariectomized and received a subcutaneous estradiol implant. These rats were sacrificed at various time periods after estradiol implantation. At the time of sacrifice, pituitaries of these animals were inspected for tumor and the whole body were inspected for any tumor metastasis. Fetal alcohol exposed animals showed increased levels of pituitary weight, pituitary prolactin (PRL), plasma PRL, pituitary aromatase, pituitary αESR and plasma estrogen as compared to those in control AD and PF rats. Estradiol

treatment also time-dependently increased pituitary weight in AF group as compared to AD and PF groups. After 90 days of estradiol treatment, inspection of the pituitary revealed that most tumors in the AF group were hemorrhagic and showing expansion to the surrounding tissue. AD and PF rats did not show any non-pituitary site tumors. Histopathological evaluation revealed that tumors in AF group were more densely packed cells as compare to the PF and AD groups which showed uniform cells with abundant cytoplasm. Pituitary tumor from alcohol exposed animals showed strong nuclear p53 and Ki67 expression. Significantly higher mRNA levels of hemorrhage-associated genes and proteins (PTTG, FGF4 and MMP-9) and multipotency genes and proteins (SOX2, Oct4 and CD133) were also observed in pituitary tumor tissues from AF group as compared with PF and AD groups. These data provide evidence for the development of aggressive and possible neoplastic prolactinomas in the pituitary after estrogen treatment in fetal alcohol exposed female rats.

Introduction

The National Toxicology Program has established that alcoholic beverages are human teratogens or human carcinogens (Doi, et al. 2004; National Toxicology 1995). Moreover, the International Association of Research on Cancer's 2009 survey estimates that around 3.5% of cancer deaths in the U.S. are caused by alcohol consumption (Nelson, et al. 2013). Epidemiological studies also identified that early environmental exposure of alcohol and drug abuse increases childhood diseases including neuropsychological disorders, cognitive deficiencies, facial abnormalities, and decreases in normal brain size, diabetes, obesity and cancer development (Blystone, et al. 2009; Hilakivi-Clarke 1997a, b, c; Hilakivi-Clarke, et al. 1997a; Kue Young, et al. 2002; Rider, et al. 2009; Wigle, et al. 2008; Young, et al. 2002). Although evidence for an increased incidence of cancer in FAS is not abundant, two reports have identified FAS children that have developed neuroblastoma under age 7, (Kinney, et al. 1980; Seeler, et al. 1979). Using animal models Hilakivi-Clarke and colleagues have shown that prenatal alcohol exposure increases the risk for mammary tumor development and that this may be due to, increases in plasma estrogen levels in the offspring (Hankinson et al. 1995; Hilakivi-Clarke 1997a; Hilakivi-Clarke, et al. 1997b). In agreement with this, Polanco and colleagues have demonstrated increased mammary tumor development in fetal alcohol exposed offspring after 16 weeks of a single NMU injection (Hilakivi-Clarke, et al. 2004). However, no studies have investigated if fetal alcohol exposure affects prolactinoma development. In this study, we tested whether FAE offspring are more susceptible to developing prolactinomas under the influence of estradiol.

The ovarian steroid estradiol is known to increase proliferation of lactotroph cells in humans as well as in laboratory animals (De Nicola, et al. 1978; Garcia and Kapcala 1995; Gomez, et al. 1977; Gooren, et al. 1988; Lloyd 1983; Sarkar, et al. 1982; Wiklund, et al. 1981). Certain populations of humans are more susceptible to estradiol's mitogenic action on lactotropes (Luciano, et al. 1985). Similarly, different strains of laboratory rats exhibit differences in lactotropic cell susceptibility to estradiol. For example, Fischer-344 (F344) (Banerjee, et al. 1994) and AxC-Irish strains (Stone, et al. 1979) are sensitive to estrogen's growth-promoting and tumor-inducing actions on the pituitary. The F344 strain is most sensitive to estrogen, and chronic estradiol treatment in this strain induces lactotropic proliferation that results in lactotropic tumors within a few months (Pastorcic, et al. 1995). Unlike F344 rats, Sprague Dawley (SD), Brown Norway, and Holzman strains show low lactotropic cell proliferation upon chronic estrogen treatment (Banerjee et al. 1994; Hokfelt, et al. 1990; Wiklund and Gorski 1982). Therefore in this study we used F344 rats. We report here that fetal alcohol exposure promotes the development of invasive prolactinomas possibly by increasing the stem cell niche.

Materials and Methods

Animals – general care

Fisher-344 rats were obtained from Harlan Laboratories (Indianapolis, IN) and housed under controlled conditions with a 12 h light/dark cycle at a constant temperature of 22°C. All animal procedures were approved by the Rutgers University Institutional Animal Care and Use Committee according to NIH guidelines. Rats were housed two animals per Open-type Shoe Box Cages with Bedcob bedding and were fed with ad libitum rat chow and tap water in a conventional facility. Health status of animals was checked regularly by determining body weight, feeding and general behaviors, and university veterinarians were consulted to address any special health needs.

Fetal alcohol exposure studies

Animals were checked for normal estrus cycle prior to breeding. Gestational status was checked the morning following mating by examining a vaginal smear on a light microscope; if sperm appeared, rats were considered pregnant and this was counted as the first day of pregnancy. On gestational day 7 through 21 rats were fed either rat chow ad libitum (AD), a liquid diet containing ethanol (AF; Bioserve, Frenchtown NJ) ad libitum or pair-fed (PF; Bioserve) an isocaloric liquid control diet (with ethanol calories replaced by maltose-dextrin). The concentration of ethanol varied in the diet for the first 4 days from 1.7 to 5.0% v/v to habituate the dams to the alcohol diet. After this habituation period, dams were fed the liquid diet containing ethanol at a concentration of 6.7% v/v. Previous studies have shown that the peak blood ethanol concentration is achieved in the range of 120–150 mg/dl (0.12–0.15%) in pregnant dams fed with this liquid diet (Miller

1992). At postnatal day 2 (PD2), AF and PF pups were cross-fostered to untreated lactating AD dams to prevent any compromised nurturing by the AF and PF moms. Litter size was maintained at 8 pups per dam to minimize any nurturing effect on the body growth. Pups were weaned on PD21, and housed by sex. In each experimental group only one female offspring from each litter was used in order to avoid any gene homogeneity.

Study using intact female rats

To determine whether FAE alters pituitary lactotropic cell growth, proliferation, and pituitary gene expression during adulthood, a group of 18 to 24 AD, PF or AF female offspring were used. Rats were sacrificed by decapitation at 90 days of age on the day of estrus (6-8 animals/feeding group). Pituitary tissues and trunk blood samples were collected for pituitary weight and hormone and gene measurements. Some of the pituitary tissues were fixed with formalin and used for IHC analyses.

Study using ovariectomized and estradiol-treated female rats

We also determined if FAE alters the mitogenic effects of estrogen on lactotropes. Estrogen is known to increase the proliferation of PRL producing lactotropic cells and the development of prolactinomas (1-7). For this, a group of 48 animals at 60 days of age was ovariectomized under sodium pentobarbital anesthesia (50–60 mg/kg, i.p.) as a general anesthesia and 2.5% Bupivacaine (sc) as a local analgesia, and then subcutaneously implanted with an estradiol-17β (Sigma, St. Louis, MO) filled 1-cm silastic capsule (Dow Corning, Midland, MI) (8 animals/feeding group) or an empty 1-cm silastic capsule (8 animals/feeding group). After surgery, animals were kept under observation for pain and suffering or infection for 3 days. Estrogen-treated rats were kept

in a cage fit with a HEPA filter to protect the user from estrogen contamination from these animals. The estradiol capsules were shown to maintain plasma levels of estradiol-17β between 120 and 150 pg/ml and induce prolactinomas (De, et al. 1996). In order to determine the time-course of estradiol effects on pituitary weight and hormone production, groups of rats were sacrificed by decapitation on 60 days, 90 days, and 120 days after the implants.

Tissue Histology

Pituitary tissues were fixed in 10% neutral-buffered formalin, and dehydrated, cleared, and embedded in Paraplast using facilities located in the Histopathology Core of the Environmental Occupational Health Sciences Institute at Rutgers University. Samples were sectioned at 6 μm and placed on slides. For pituitaries, cross sections were obtained. Sections were stained with hematoxylin and eosin using conventional protocol and mounted with Permount. An experienced pathologist, Dr. Kenneth Reuhl, who was blind to treatment, viewed tumor slides to assess tissue pathology. Representative images were taken using a Nikon microscope at 10X and 20X.

Immunohistochemistry (IHC)

Slides containing 6 μm pituitary sections were baked overnight at 60°C. Sections were then deparaffinized in xylene and rehydrated in decreasing concentrations of ethanol. Antigen retrieval was performed by heating the slides in 10 mM sodium citrate (pH 6.0) at 95°C for 20 min then cooled to room temperature. All antibody and vendor information is listed in Table 1. In general, tissues were blocked in normal horse serum (Vector, Burlingame, CA) for 60 minutes at room temperature. Samples were incubated

overnight at 4°C with either primary antibody or rabbit primary antibody isotype control (Life Technologies; Grand Island, NY), which served as a negative control for each slide. The following day the tissues were incubated with secondary antibody (Vector, Burlingame, CA) for 60 minutes at room temperature. Slides were counterstained with DAPI (Vector, SK-4100) then mounted with Permount Prolong media. For all IHC, tumor sections were viewed and five representative pictures of each section were taken at random using an Olympus FSX100 microscope at 20X (Olympus). The pictures were taken with the same exposure settings for all samples. The amounts of Ki67, P53, FGF4, PTTG, MMP9, SOX2, CD133, OCT4 protein staining in each section were counted using Photoshop software, only dark brown staining was counted as positive staining. The number of stained cells and number of total cells (hematoxylin stained cells) within a 4000 μm area was counted and the percent of cells stained was calculated by dividing the number of positive cells with the number of total cells x 100.

Enzyme linked immunosorbent assays (ELISA) for PRL and estrogen

Plasma PRL and estradiol levels were measured using a rat PRL ELISA kit (Alpco Diagnostics, 55-PRLRT-E01, Salem, NH) and rat estrogen ELISA kit (My BioSource, MBS703614; San Diego, CA) respectively, as per the instructions from the manufacturer.

Quantitative PCR for gene expression measurements

Gene expression levels of aromatase, αESR, Ki67, P53, FGF4, PTTG, MMP9, SOX2, CD133, OCT4 in rat pituitaries were measured by quantitative PCR (SYBR green assay). Total RNA from pituitary glands was extracted using the All in One Purification Kit (Norgen Biotek, Ontario, Canada). Total RNA (1 μg) was converted to first strand

complementary DNA (cDNA) using a high capacity cDNA reverse transcription kit (Applied Biosystems, Carlsbad, CA). All the primer sequences used for the study are given in Table 2. Quantitative PCR was performed at 95°C for 5 min followed by 40 cycles of 95°C for 15 sec, 60°C for 30 sec, 72°C for 40 sec using Applied Biosystems 7500 Real time PCR system. The quantity of target gene expression was measured using the standard curve method. GAPDH and ribosomal protein 19 (RPL-19) were used as internal controls. GAPDH and RPL-19 did not vary between groups. Gene expression levels as GAPDH ratios are presented in the figures.

Statistical analysis

Data were analyzed using Prism 5.0 (GraphPad) Software, and presented in the figures as mean ± SEM. The significant differences between different treatment groups were assessed with one-way analysis of variance (ANOVA) and Newman Keuls posttest. $P<0.05$ was considered significant.

Results

1- Fetal alcohol exposure increases pituitary weights and levels of pituitary prolactin (PRL), plasma PRL, pituitary aromatase, and plasma estrogen in female offspring

Previous studies have reported that chronic or binge alcohol drinking increases plasma PRL and estrogen levels in humans and in various animals models (Hilakivi-Clarke et al. 2004; Mello et al. 1988; Mennella and Pepino 2006, 2008). Our laboratory has recently shown that fetal alcohol exposure (FAE) increases the incidence of prolactinomas (Gangisetty, et al. 2015). The underlying mechanisms for increased susceptibility to tumorigenesis in the pituitary of these offspring are not apparent. Many human tissues

express the enzyme aromatase, which locally catalyzes the conversion of steroids to estrogens (Gao, et al. 2017). There is increasing evidence that aromatase may be involved in some tumor cell proliferation pathways. Hence, we hypothesized that aromatase expression is increased in the pituitary of FAE animals, providing a local source of estrogens to stimulate the proliferation of prolactin-producing lactotrophs. To test this we measured the pituitary content for PRL and aromatase and by single or double-staining IHC procedures. We also measured aromatase and alpha estrogen receptor mRNA levels in these tissues using quantitative RT-PCR methods. Additionally, we measured plasma levels of PRL and estrogen by ELISAs and pituitary wet weight. As expected from the previous work (Gangisetty et al. 2015), pituitary PRL immunoreactivity was increased in AF animals (Fig. 1A, B). The IHC staining data also showed increased cells with positive staining for aromatase in FAE animal pituitary tissues (Fig. 1C, D). We found that the level of PRL mRNA and aromatase mRNA, but not αESR mRNA, was significantly higher in fetal alcohol-fed animals, as compared to controls (Fig. 1E, F, G). Additionally, we found that plasma estrogen levels (H), plasma PRL levels (I) and pituitary weights were higher in fetal alcohol-fed animals as compared to controls. These data provide evidence supporting the hypothesis that estrogen overproduction in the pituitary and other tissues in the periphery increases the susceptibility of PRL-producing lactotropes to develop tumors.

2- Fetal alcohol exposure increases the development of aggressive prolactinomas in the pituitary after estrogen treatment in female rats

The experiment described above indicates that in utero alcohol exposure enhances plasma and pituitary estrogen levels in the offspring. Because estrogen is mitogenic to

lactotropes (De Nicola et al. 1978; Gomez et al. 1977; Landolt, et al. 1987), we hypothesized that prolonged elevation of estrogen production in FAE offspring may lead to the development of more aggressive pituitary tumors. There is evidence that estrogen produced locally through aromatization might enhance tissue expression of PTTG and FGF2 which are known to be markers of an aggressive prolactinoma (Yilmaz, et al. 2015). To address this, we first compared the time-dependent effects of estrogen on the pituitary weight (as a measure of tumor development) and the plasma PRL level (as a measure of hormone secreted by the tumor) in fetal alcohol-exposed and control diet-exposed offspring. These data are shown in Figure 2. Estrogen treatment via a silastic capsule that maintained plasma estrogen levels at 120 pg/ml increased both pituitary weights and plasma levels of PRL. Estrogen effects on pituitary weights and plasma PRL levels were time-dependent; the longer the treatment the higher the pituitary weight and PRL level (compare data between 2A and E). It is also important to note that both pituitary weight and PRL responses to estrogen were higher in AF group than those in AD and PF groups at all time points. Inspection of the pituitary gland 120 days after estrogen treatment revealed that some of the pituitary tumors of AF animals were highly vascularized, penetrating to the sphenoid bone and pressing on the median eminence (Fig. 2G top two panels). Pituitaries of estrogen-treated AD and PF rats were smaller and less vascularized (Fig. 2G bottom two panels). Histopathological inspections showed that pituitaries of AF animals had small round tumor cells in a solid pattern, necrosis, and the epithelial cells colonized in nested shape surrounded with blood vessels, which indicates angiogenesis signaling, while the pituitary tumor section from AD and PF animals showed uniform epithelial cells and infiltration of eosinophilic cells (Figure 2H). These

data suggest that FAE rat pituitaries, particularly PRL producing lactotropes, are more responsive to the mitogenic action of estrogen.

3- Fetal alcohol exposure increases the expression of Ki67 and other biomarkers of cell proliferation in the pituitary tumor tissue

In order to further determine the aggressiveness of pituitary tumors, various biomarkers for aggressive pituitary adenomas were studied. As discussed earlier pituitary adenomas exhibit a wide range of behaviors and the prediction of aggressive or malignant behavior in pituitary adenomas remains challenging. Recent evidence suggests that the expression of FGFR4, MMP, PTTG, Ki-67, p53 may serve as biomarkers for aggressive pituitary adenomas (Mete et al. 2012).

In order to determine the characteristic changes in pituitary tumors of FAE rats, the Ki67 and P53 labeling indices were first examined by staining the pituitary tumor tissue from AF, PF and AD rats. As shown in Fig. 3A and B, Ki67 immuno-labeling was higher in the pituitary of FAE rats. Similarly, the nuclear P53 immuno-labeling was also elevated in the pituitary of FAE rats (3C and D).

The expression levels of various oncogenes (FGFR4, PTTG and MMP9) in the pituitaries were also determined. FGFR4 is a member of the FGF family, which plays an important role in the pituitary gland organogenesis, proliferation of the pituitary progenitor cells during embryonic development, and is widely expressed in human invasive pituitary adenoma (Abbass, et al. 1997; Ericson, et al. 1998; Ezzat, et al. 2002; Norlin, et al. 2000; Qian, et al. 2004). PTTG is an integrin heterodimeric receptor and plays a beneficial role in gathering the cell membrane with the extracellular matrix.

Overexpression of PTTG protein has been found in a variety of cancers such as colorectal cancer, breast cancer and invasive ovarian cancer. PTTG has also been identified to play a role in prolactinoma formation (Fong, et al. 2012; Gao, et al. 2016; Horwitz, et al. 2003; Panguluri, et al. 2008; Salehi et al. 2008; Shah, et al. 2012; Zhou, et al. 2014). Matrix metalloproteinase (MMPs) are zinc dependent proteinase enzymes that contribute to tissue homeostasis, organ development, and cancer progression by digestion of extracellular matrix to allow tumor cells to penetrate the basement membrane and migrate to other parts of the body. Some studies have shown that the invasive grade of human pituitary adenoma overexpresses MMP9. Furthermore, MMP9 overexpression has been found in several types of metastatic cancer such as liver cancer, breast cancer and ovarian cancer (Gong, et al. 2008; Gultekin, et al. 2015; Hussaini, et al. 2007; Kamat, et al. 2006; Mete et al. 2012; Pellikainen, et al. 2004; Shchors, et al. 2013; Sillanpaa, et al. 2007). We measured the expression of these oncogenes in the pituitary by using both immunostaining procedures for protein and real-time PCR procedures for gene expression. As can be seen in Figure 3, FAE pituitaries had increased protein (E, F) and gene expression levels of FGFR4 (G) as compared to control rat pituitaries. Fetal alcohol-exposed pituitaries also showed increased protein and mRNA levels of PTTG (Fig. 3H, I, J) and MMP9 (Fig. 3K, L, M). These biochemical data together with histopathological data shown in Fig. 2 support the notion that FAE rat pituitaries develop aggressive prolactinomas following estrogen treatment.

4- Identification of stem-like cells in the pituitary tumors of fetal alcohol-exposed rats

The presence of adult pituitary stem cells (PSCs) has been described in murine systems by comprehensive cellular profiling and genetic lineage tracing experiments. PSCs are thought to maintain multipotent capacity throughout life and give rise to all hormone-producing cell lineages, playing a role in pituitary gland homeostasis. Additionally, PSCs have been proposed to play a role in pituitary tumorigenesis, in both adenomas and adamantinomatous craniopharyngiomas (Gao et al. 2017; Manoranjan, et al. 2016). Recently, a role for PSCs in the formation of aggressive pituitary tumors has been proposed (Garcia and Kapcala 1995; Luciano et al. 1985) In this study, pituitary tumor sections from AF, PF and AD rats were stained for the stem cell markers SOX2, CD133 and OCT4 and corresponding mRNA levels were measured by qRT-PCR. The pituitary tumor sections from AF animals demonstrated increased staining for SOX2, CD133 and OCT4 as compared to those in control groups (Fig. 4A, B, D, E, G, H). Fetal-alcohol exposed pituitaries also showed increased mRNA levels of SOX2 (Fig. 4C), CD133 (Fig. 4E) and OCT4 (Fig. 4H). These data suggest that pituitary tumors of FAE rats contain more PSCs.

Discussion

Overall, the data presented here indicate that FAE results in increased levels of pituitary weights, pituitary PRL and plasma PRL, reflecting greater growth of prolactinomas in FAE offspring exposed to estrogen. The higher growth of prolactinomas in FAE offspring could be related partly to increased production of estrogen, as we have

found increased levels of pituitary aromatase and plasma estrogen in AF rats. We also found that long-term estrogen treatment induces large, hemorrhagic and spreading tumors, higher levels of hemorrhage-associated genes and proteins (PTTG, FGF4 and MMP-9) and multipotency genes and proteins (SOX2, Oct4 and CD133) in tumors of AF rats. These data provide evidence for the development of aggressive prolactinomas in the pituitary after estrogen treatment in FAE female rats.

Pituitary tumors are the second most common intracranial tumor and account for around 20% of all types of intracranial tumors (Meij et al. 2002; Mete et al. 2012; Selman, et al. 1986). Although pituitary tumors are usually benign and treatable, their invasiveness and aggressiveness behavior poses a major challenge for clinicians. Even with new surgical techniques such as radiological and MRI diagnoses, histological findings and some biochemical analyses, it is hard to detect the penetration of the tumor cells to the surrounding tissue such as dura, nasal cavity and diaphragm. Furthermore, some pituitary tumors have inheritance features to relapse and regrow in more aggressive patterns or may show metastatic behavior (Lillehei, et al. 1998; Meij et al. 2002; Mete et al. 2012; Partington, et al. 1994). Since pituitary tumors are commonly benign, we predict that maternal alcohol exposure reprograms the pituitary to develop aggressive tumors in the offspring. Our conclusion that pituitary tumors of FAE rats are more responsive to develop aggressive pituitary tumors is based on the following: First, FAE pituitaries are large and hemorrhagic. The macroscopic appearance of pituitary tumor from AF animals represents soft and loosely formed tumor as compared to the control groups. Second, we have used most of the reliable IHC markers that characterize aggressive pituitary adenoma. For example, we used Ki67 as a marker that is a nuclear antigen and expresses

in the S, G1, G2 and M phase, but not in the G0 phase or in quiescence cells. Increased Ki67 expression might be associated with metastatic and malignant variety of cancers (Gerdes, et al. 1984; Parkins, et al. 1991a; Parkins, et al. 1991b). The reliability of Ki67 labeling index in pituitary tumor is different from other tumor types. The Ki67 labeling index varies between <1% to as high as 23% (Salehi et al. 2009) and counting 3% and higher as the threshold to diagnose the pituitary invasiveness. Our data reveals that Ki67 labeling index was >4. A strong correlation between Ki67 labeling index and pituitary invasive adenoma with 97% specificity and 73% sensitivity has been found in several studies (Daita and Yonemasu 1996; Iuchi, et al. 2000; Jaffrain-Rea, et al. 2002; Landolt et al. 1987; Thapar et al. 1996a; Wolfsberger, et al. 2004; Zhao, et al. 1999). Thus, these data strongly support our prediction that FAE enhances invasive pituitary adenoma. We also used P53 as one of the biomarkers of aggressive pituitary tumors. P53 labeling index is well known as an indicator for malignancy and metastasis in different types of cancers. In a study examining the relationship between P53 expression and invasiveness, P53 expression was nondetectable in non-invasive adenoma, 15% in invasive adenoma and 100% in carcinoma (Hentschel, et al. 2003; Thapar et al. 1996b). In our experiment, we found FAE pituitaries have P53 labeling index <30, which also supports our notion that FAE increases the incidence of invasive pituitary adenoma. The pituitary tumor tissues of offspring exposed to alcohol during embryonic life also expressed higher levels of the oncogenes PTTG, FGF4 and MMP-9, PTTG is multifunctional pro-oncogene that plays several functions in tumor development such as cell transformation, DNA repair, DNA replication, angiogenesis and control of gene expression. Overexpression of PTTG in tumor tissue correlates strongly with cancer invasion, metastases and poor prognosis

(Dominguez, et al. 1998; Hamid, et al. 2005; Shibata, et al. 2002; Solbach et al. 2004). PTTG overexpression through nuclear binding factors leads to activated FGF expression, which positively correlates with the invasion of pituitary adenoma (Horwitz et al. 2003; McCabe, et al. 2003). In addition, MMP9, a proteolytic enzyme known to be upregulated in invasive prolactinoma and pituitary carcinoma (Ceylan, et al. 2011; Gong et al. 2008; Kawamoto, et al. 1996a; Kawamoto, et al. 1996b; Liu, et al. 2005; Paez Pereda, et al. 2000; Turner, et al. 2000a) is also upregulated in the pituitary of FAE offspring. Hence, increased MMP9, PTTG and FGFR4 levels in the tumor identify an aggressive phenotype.

Recently, a role for stem cells in the formation of aggressive pituitary tumors has been proposed. Pituitary stem cells expressing the transcription factor SOX2 are able to contribute to the generation of new hormone-producing cells during postnatal life. Furthermore, it has been shown in mice with forced up-regulation of the SOX2-positive pituitary stem cells by transgenic approaches stimulates a transient burst of proliferation, and subsequently induces tumorigenesis in a non-cell autonomous manner (Andoniadou 2016). Recent studies also showed that cells expressing CD133 in pituitary adenomas partially exhibit stem cell properties (Xu et al. 2009). Oct4 and Sox2 are two typical embryonic stem cell markers and are established markers of pituitary stem cells (Chang, et al. 2017). Their existence in pituitary adenomas has also been demonstrated (Gao et al. 2017; Garcia-Lavandeira et al. 2012; Orciani et al. 2015). Additionally, rat prolactinoma cells also contained OCT4- and SOX2-positive cells (Gao et al. 2017). Therefore, our results showing the increase in SOX2, CD133 and OCT-4 in the pituitary tumors of FAE

rats suggest that these cells express stem cell markers which may contribute to their aggressiveness.

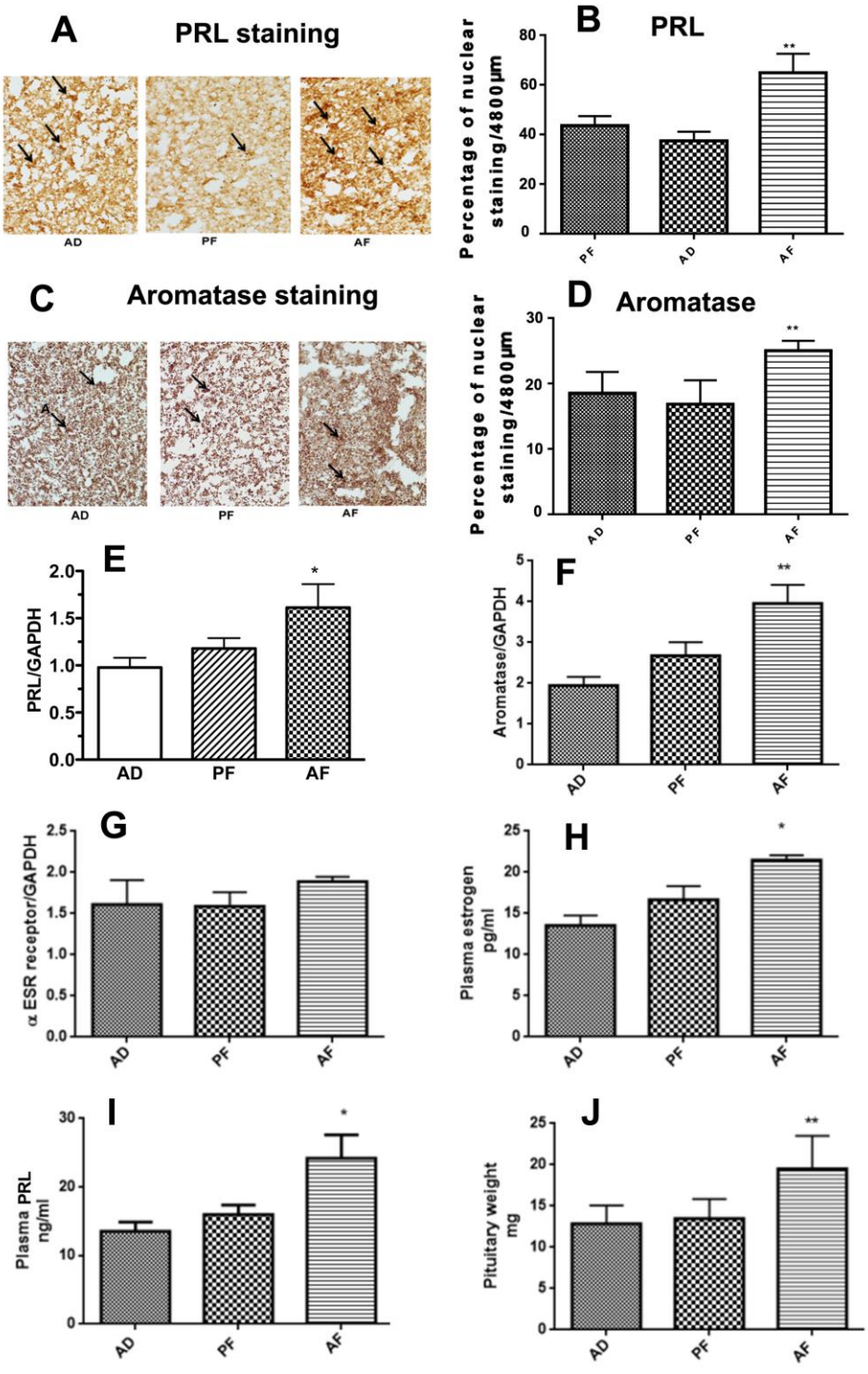
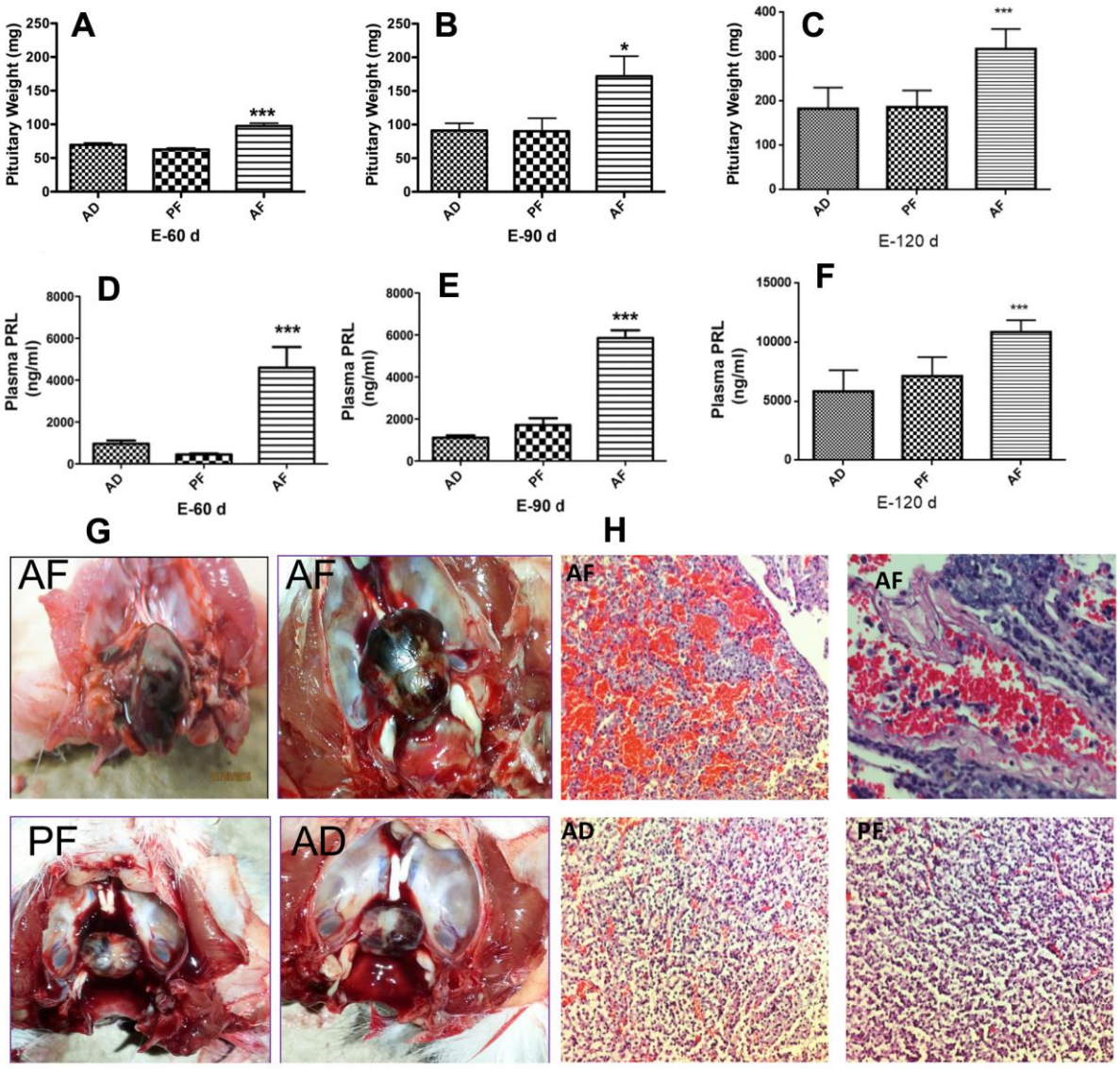

A
PRL staining
AD
PF
AF
B
PRL
Percentage of nuclear staining/4800μm
C
Aromatase staining
D
Aromatase
E
PRL/GAPDH
F
Aromatase/GAPDH
G
α ESR receptor/GAPDH
H
Plasma estrogen pg/ml
I
Plasma PRL ng/ml
J
Pituitary weight mg

Figure 1. Effects of fetal alcohol exposure on the immunoreactivity of pituitary prolactin (PRL) (A, B) and aromatase (C, D), pituitary PRL mRNA level (E), aromatase mRNA level (F), plasma estrogen level (G), pituitary estrogen receptor (αESR) mRNA level (H) plasma PRL level (I) and pituitary weight (J) in female rats. Fetal alcohol-fed (AF), pair-fed (PF) and ad libitum-fed (AD) rats were used during the adult period (90 days) on the day of estrus. Data are mean ± SEM (n = 6-8) and were analyzed using one-way analysis of variance (ANOVA) with the Newman-Keul posthoc test. *, $p<0.05$, **, $p<0.01$ and ***, $p<0.001$ between AF and controls (AD, PF).


A
Pituitary Weight (mg)
250
200
150
100
50
0
***
AD
PF
AF
E-60 d
B
Pituitary Weight (mg)
250
200
150
100
50
0
*
AD
PF
AF
E-90 d
C
Pituitary Weight (mg)
400
300
200
100
0
***
AD
PF
AF
E-120 d
D
Plasma PRL (ng/ml)
8000
6000
4000
2000
0
***
AD
PF
AF
E-60 d
E
Plasma PRL (ng/ml)
8000
6000
4000
2000
0
***
AD
PF
AF
E-90 d
F
Plasma PRL (ng/ml)
15000
10000
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AD
PF
AF
E-120 d
G
H
AF
AF
AF
AF
PF
AD
AD
PF

Figure 2. Effects of fetal alcohol exposure on estrogen-induced changes in pituitary weight, plasma PRL level and pituitary histopathologies. Fetal alcohol-fed (AF) or control-fed (PF, AD) rats were ovariectomized and implanted with a β-estradiol implants at 60 days of age and used after 60 (E2-60 d), 90 days (E2-90 d) or 120 days (E2-120 d) for measurements of pituitary weight (A-C) and plasma PRL (D-F). Data are mean ± SEM (n = 6-8) and were analyzed using one-way analysis of variance (ANOVA) with the Newman-Keul posthoc test; *, $p<0.05$, **, $p<0.01$ and ***, $p<0.001$ between AF and controls (AD, PF). Representative photomicrographs of the pituitaries (G) and histopathology of tumors (H). evaluated following histological staining using Haematoxylin and Eosin by a pathologist (Magnification X10).

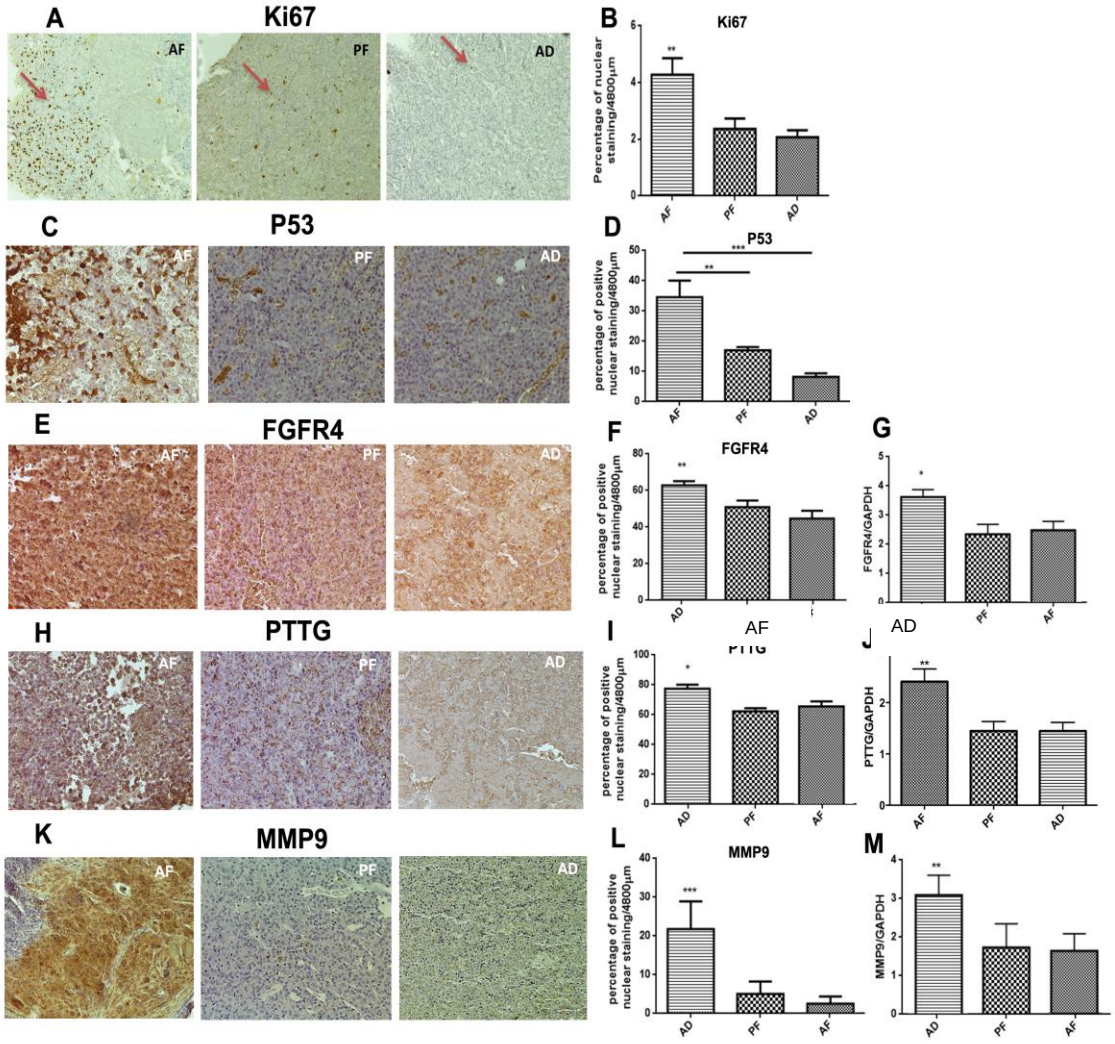

Figure 3. Characterization of the expression of tumor aggressiveness markers in the pituitary of fetal alcohol exposed rats. Pituitary tumor tissues of AF, PF and AD rats were stained for Ki67, P53, FGF4, PTTG or MMP9 using histochemical techniques. Photomicrographs of these tumor aggressiveness markers are shown on the left panels (A, C, E, H, K) and the percentage of positive stained cells shown on the right panels (B, D, F, I, L).. The magnification 10X for Ki67 and 20X for the rest (P53, FGF4, PTTG or MMP9). The levels of FGF4, PTTG and MMP9 mRNAs are shown in panels G, J and M, respectively. Data are expressed as mean ± SEM (n= 6-8) and were analyzed using one-

way analysis of variance (ANOVA) with the Tukey's multiple comparisons posttest. *, $p<0.05$, **, $p<0.01$ and ***, $p<0.001$ between AF and controls (AD, PF) or indicated by a bar on the top of the graphs.

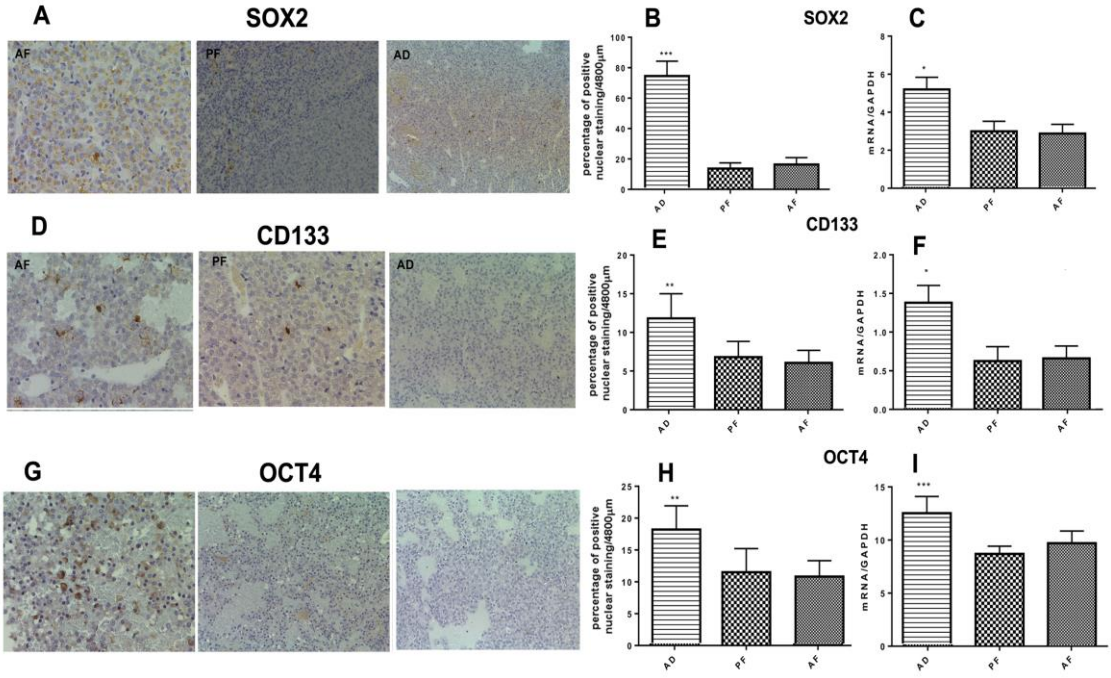

Figure 4. Characterization of the expression of pituitary stem cell (PSC) markers in the pituitary of fetal alcohol exposed rats. Photomicrographs of these PSC markers are shown on the left panels (A, D, G) and the percentage of the positively stained cells shown on the right panels (B, E, H). The pictures are 20X magnification. The level of SOX2, OCT4 or CD133 mRNAs are shown in panels C, F and I, respectively. Data are expressed as mean ± SEM (n= 6-8) and were analyzed using one-way analysis of variance (ANOVA) with the Tukey's multiple comparisons post-test. *, $p<0.05$, **, $p<0.01$ and ***, $p<0.001$.

Chapter 3

Pituitary tumors from alcohol-exposed animals contain a cell population that displays stem-like properties with high invasive and metastatic potential

Abstract

Data from the previous chapter showed that pituitaries of fetal alcohol-exposed (FAE) rats develop large prolactinomas with aggressive tumor phenotypes. In cases of malignancy and aggressive tumors, stem and progenitor–like cells (cancer stem cells; CSC) serve a critical role in the tumor microenvironment and in the processes of cancer cell proliferation, migration, invasion and angiogenesis. The evidence for the existence of CSCs in rat pituitary tumors needs to be elucidated. In this study, we tested whether FAE enhances the population of CSCs and aggressive prolactinomas in the pituitary in response to estradiol. Pregnant Fischer 344 rats were fed between gestational days 7 and 21 with a liquid diet containing alcohol (AF), or fed ad libitum with rat chow (AD). At 60 days of age, female offspring were ovariectomized and received a subcutaneous estradiol implant. Rats were sacrificed 4 months after the estradiol implants were placed. Tertiary pituispheres were generated form pituitary tissue and used for determination of stemness and tumorigenic potential. Expression levels of mRNA and protein for genes related to multipotency (OCT4, NANOG, KLF4, SOX2, CD133, CD44, nestin and CD34) were significantly higher in pituispheres of AF animals compared to expression levels in pituitspheres of AD animals. Cells generated from pituispheres isolated from AF animals showed higher cell proliferation, migration and colony formation rates as compared to control groups. These data provide evidence that FAE may potentially promote the development of aggressive pituitary tumors in estrogen-treated animals.

Introduction

Tumorigenesis is considered a multistep process and understanding the molecular pathways responsible for initiation and progression is important in developing and applying effective therapeutics (Vogelstein and Kinzler 1993). Some critical hallmarks of cancer development involve the tumor acquiring sustained proliferative signaling, replicative immortality, and activating invasion and metastasis. The acquisition of specific "hallmarks" is widely accepted to be fundamental in cancer development (Hanahan and Weinberg 2000). Acquisition of "stemness" is also an important hallmark of cancer development.

Cancer stem cells (CSCs) are a small subset of cells within a tumor that have the distinct ability to self-renew and seed the heterogeneous populations which comprise the majority of the tumor (Marsden, et al. 2009). CSCs are proposed to promote tumor initiation and progression and have been implicated in chemoresistance and tumor relapse suggesting they drive an aggressive disease state (Hanahan and Weinberg 2011). CSCs do not necessarily originate from stem cell populations in normal tissue and can come from differentiated cancer cells populations that have acquired plasticity (Visvader and Lindeman 2008). Although the origins of CSC populations are not fully understood, the activation of an epithelial-to-mesenchymal transition (EMT) has been shown to confer stem-like properties on cancer cells (Cordenonsi, et al. 2011; Lei, et al. 2008; Mani, et al. 2008; Qiao, et al. 2012; Yu, et al. 2012). EMT occurs when a cell loses its epithelial characteristics, in particular cell-cell contacts and polarity, and gains a mesenchymal phenotype, allowing the cell to escape the epithelial layer, acquire motility, evade apoptotic cues, and invade the basement membrane (Gilchrist, et al. 2002; Hay 1995;

Thiery 2002). Overexpression of factors that promote EMT, such as transcription factors Twist and Snail, or cytokines like TGFβ, can promote tumor-initiating populations (Mani et al. 2008; Pirozzi, et al. 2011; Qiao et al. 2012; Thiery 2002). CSCs can be identified and isolated by distinct cell markers, similar to normal stem cell populations, however the expression of these markers varies depending on context. In the pituitary tumor, stem cells express OCT4, Nanog, Klf4, CD44, Sox2, Sox9, CXCR4, Nestin, S100 (Chen et al. 2009; Chen et al. 2005; Gleiberman et al. 2008; Goodell, et al. 1996; Krylyshkina, et al. 2005; Lepore, et al. 2005) and correspond with tumor aggressiveness (see chapter 2 for discussion). In this chapter, we demonstrate the existence of stem/progenitor cells in the pituitary tumors of FAE rats and show that these cells have invasive and metastatic properties.

Materials and methods

Preparation of the anterior pituitary gland for cell isolation

The methods of FAE, ovariectomy and estrogen treatment have been described in Chapter 2. Fischer 344 rats were administered AD or AF treatments (6/group) and the experiment was repeated three times. Three offspring from each dam were implanted with estradiol capsules for four months to promote pituitary tumor formation. The animals were killed by decapitation. Under sterile conditions in the hood the skull was removed using a bone cutter and placed in a sterile dish containing sterile 1X PBS. The gland was cut into two transverse sections. One-half was placed into a specimen cup and processed for IHC as described below. The anterior pituitary gland was excised from the other half and put in a 15 ml conical tube containing 10 ml HBSS with reduced calcium (Sigma-Aldrich; catalog #H6136) for further analyses as described below.

Isolation and culture of pituitary cells

The pituitary cell culture method was adapted from several published methods (Andoniadou et al. 2012; Chen et al. 2005; Fauquier et al. 2008; Gaston-Massuet et al. 2011; Gleiberman et al. 2008). Pituitary tissue from three offspring per dam was combined. The tissue was first washed several times to remove all the blood and then digested using collagenase (0.5%; Sigma-Aldrich; catalog #C9891) and DNAase (0.5%; Sigma-Aldrich; catalog #D4263) in HBSS for 15 min at 37ºC with frequent pipetting using a glass pasture pipette. The reaction was stopped by adding equal volumes of DMEM/F12 medium (Sigma-Aldrich; catalog #D2906) with 5% FBS (Gibco). The suspension was centrifuged at 3000 rpm for five minutes to collect the pellet. Each pituitary isolation representing tissue from three offspring yielded more than 500,000 cells and the viability was >85%. Cell counting was done using a hemocytometer and the cell viability was measured by methylene blue exclusion.

Generation of pituispheres

In order to determine if the pituitary cells have stem cell-like properties, the freshly dissociated cell pellet was prepared in growth medium (10 cells/µl) and plated on an ultralow attachment plate (9.5 cm^2; Corning 6 wells plates; catalog #3471) at 500 µl/well. Cells were maintained at 37ºC and 5% CO_2. Growth medium contained DMEM/F12 medium, 0.5% BSA, B27 serum supplement (1:50; Life technology) and bFGF (20 ng/ml; Sigma), EGF (20 ng/ml; Sigma), glutamine (200 mM; Life Technology) and N2-supplements (Life technology). The culture was fed by adding small amounts of fresh growth medium every day, and the medium was changed every 3 days. After 7-14 days,

the spheres started growing. Spheres were trypsinized after another 7–14 days and passaged to evaluate secondary sphere formation. The cells from the dissociated spheres were collected in a 15 ml conical tube and centrifuged at 3000 rpm for 5 minutes. Then 3 ml warm filtered trypsin (0.25% EDTA trypsin; Gibco Invitrogen) was added and tubes were kept in a water bath at 37ºC for 5 minute then mechanically dissociated by using a glass pasture pipette or 1 ml pipette. The trypsin reaction was stopped by adding an equal volume of DMEM/F12 medium with 0.5% BSA. Sometimes the spheres were big and hard to dissociate. In such cases spheres were incubated with cold trypsin at 4ºC for 20-25 min and then mechanically dissociated using a pasture pipette, and trypsin reaction stopped by adding equal volume of DMEM/F12 with 0.5% BSA. The cell suspension was spun down at 3000 rpm for five minutes, cell pellet was collected and cells were plated in a new ultralow attachment 6 well plate at 2000-2500 cells/well. This process was repeated with secondary spheres to generate tertiary spheres. These cells were trypsinized and used for in vitro and in vivo assays described below. A portion of the cells were frozen down and stored in liquid nitrogen. Tertiary spheres were successfully generated with pituitary cells from all 12 animals.

Immunofluorescent staining (IFC)

Pituitary cells derived from tertiary pituispheres were plated on lab-tech chamber slides and stained for various stem cell markers using IFC. Cells on culture slides were first washed three times with cold 1X PBS for removing culture medium, fixed with cold 4% PFA for 30 min, washed three times with 1X PBS and permeabilized and blocked with Triton X-100 (0.5% and 2.5% Horse serum in PBS) for one hour. Cells were then incubated with primary antibody (antibody information is listed in Table 1) overnight at

4C, washed three times with 1X PBS, and incubated with either Alexafluor 488 or Alexafluor 594 secondary antibody (Life Technologies) for 60 minutes at room temperature. Cells were counterstained with DAPI (Vector Laboratories) and imaged using a Nikon fluorescence microscope.

Cell proliferation by BrdU growth assay

Proliferation of cells isolated from tertiary pituispheres was tested using a BrdU Cell Proliferation Assay Kit #6813 (Cell Signaling Technology, Boston, MA) according to the manufacturer's protocol. 2500 cells per well were plated on a 96-well plate in quadruplicate for each pituisphere isolation.

Soft agar colony formation assay

To assess tumor cell colony formation, the CytoSelect 96-Well Cell Transformation Assay CBA-130 (Cell Biolabs, Inc. San Diego, CA) was used according to the manufacturer's instructions. Briefly, cells derived from pituispheres were seeded in soft agar at a density of 1000 cells per well in the presence of growth factors in triplicate. After 7 days of incubation at 37°C in 5% CO_2, colony formation was quantified by solubilizing soft agar, lysing cells, and incubating cell lysates with the CyQUANT GR Dye (Cell Biolabs Inc.). Fluorescence was measured using a TECAN fluorescent reader with a 485/538 nm filter set and 530 nm cutoff. The number of transformed cells was counted according to the manufacturer's instructions. Some wells were used for staining after seven days. In this case, wells were washed with 1X PBS and fixed with 4% PFA for 30 minutes, then washed three times with 1X PBS, stained using crystal violet, and visualized with the Nikon microscope.

Cell migration and invasion assays

To assess tumor cell migration, the CytoSelect 96-well cell migration assay kit CBA-106 (Cell Biolabs Inc.) was used according to the manufacturer's protocol. Briefly, cells were suspended in DMEM-serum free medium, and 10,000 cells were added to the top chambers of the 96-well cell migration plates. Five replicate wells were plated for each pituisphere isolation. Complete media was added to the bottom chambers as attractant. Twenty-four hours after incubation, migrating cells were detached from the underside of the membrane using cell detachment solution, lysed with lysis buffer and stained with CyQuant GR dye solution. Fluorescence intensity was determined with a TECONIC plate reader at 485/535 nm.

Tumor xenograft study

To determine if the pituitary cells derived from tertiary spheres could form tumors in vivo, NOD/SCID mice were used. After trypsinization of the AF and AD spheres, the cells were mixed with equal volumes of Matrigel Basement Membrane Matrix cat# 356237. The final injection volume was 200 µl/2X10^6. Mice were transplanted from each of six cell preparations prepared from the pituitary tissue of AF and each of five cell preparations prepared from AD animals. After the tumors start growing, tumor volumes were measured every other day. Tumor long length (L) and short length (S) were measured using a Vernier caliper and tumor volume was calculated using the formula (S^2 X L/2) X body weight.

Results

1. Fetal alcohol exposed pituitaries contain higher numbers of aggressive and proliferative stem-like cells

Pituitary tissue from AF (n=6) and AD animals (n=5) were dissociated and cultured on ultralow attachment plates to track progenitor/stem cell characteristics. Under these conditions, pituitary cells rapidly (within 7 days) formed spheres (Fig. 1 A). Cells isolated from both AD and AF tertiary pituispheres were able to grow rapidly in culture conditions, but the growth rate of cells isolated from AF pituispheres was several-fold greater than that of cells isolated from AD pituispheres (Fig. 1B). To determine whether these cells express stem cell-related transcription factors, we measured mRNA levels of Sox2, NANOG, OCT4, CD44, CD133, Nestin, CD34 and Klf4 genes. Pituispheres of both AF and AD rats expressed all stem cell-related transcription factors, but mRNA levels of these genes were higher in AF cells than those in AD cells (Fig. 1C). Immunostaining also demonstrated elevated expression of stem cell-related transcription factors in AF pituispheres compared with AD pituispheres (Fig. 1D and E). These data suggest that estrogen-induced pituitary tumors display progenitor or stem-like properties, and further indicate that pituitary tissue from FAE animals contain more proliferative stem-like cells.

2- Pituispheres of fetal alcohol exposed rats have significant migration and metastatic abilities and tumorigenic properties

The colony formation assay is one of the in vitro assays that examine the ability of cells to form colonies in semisolid agar. We compared the ability of pituitary cells

derived from pituispheres of AF and AD rats to form colonies in soft agar under normal growth conditions. As shown in Fig. 2A, cells derived from pituispheres of AF rats formed colonies at a four-fold higher rate than those of AD rats, suggesting that these cells have a signature of aggressive behavior. We have also determined the invasive and migration properties of the pituisphere-derived cells using the Boyden chamber assay, which mimics the ability of the cells to penetrate the extracellular matrix to migrate to others part of the organ or body. Figure 2A shows that migration rates of AD cells were very low, while migration rates of AF tumor cells were many fold higher (Fig. 2B), suggesting that AF cells from pituispheres cells have high migration ability.

It has been suggested that tumor progenitor cells or stem-like cells cannot be considered as cancer stem cells unless they are able to sustain the neoplasm (Hanahan and Coussens 2012; Hanahan and Weinberg 2011; Kreso and Dick 2014). Therefore, we studied tumor growth potential of AF and AD cells cultured from pituispheres in immunodeficient mice (NOD/SCID). In fact, AF tumor cells, but not AD tumor cells, successfully generated tumors in immunodeficient hosts (Fig. 2C). Additionally, similar to results of in vitro (Fig. 1B) assays, cells cultured from AF pituispheres rapidly grew in immunodeficient hosts (Fig. 2D). These data suggest that AF tumor cells have cancer stem cell-like properties and have the ability to renew and generate new tumor bulk in immunodeficient hosts.

Discussion

In this study we have shown that pituitary tissue obtained from Fischer 344 rats exposed to alcohol in utero and treated with chronic estrogen treatment contains a cell population that gives rise to pituispheres under in vitro conditions. Also, pituispheres, particularly from FAE rats, express higher levels of various stem cell and tumor aggressiveness markers. Cells isolated from pituispheres of AF rats had the ability to self-renew and exhibited rapid proliferation. Furthermore, they were able to form colonies and migrate under in vitro conditions. Cells generated from AF pituispheres also successfully generated tumors when transplanted in NOD/SCID mice. These data suggest that FAE promotes the development of a stem cell niche within the pituitary tumor to increase the aggressiveness of the tumor.

Previous studies have shown the existence of pituitary stem cells or pituitary progenitor cells in pituitary adenomas (Chen et al. 2014; Florio 2011; Tunici and Yu 2009; Zhou, et al. 2013). Using known progenitor/stem cell markers like Sox2, NANOG, OCT4, CD44, CD133, Nestin, CD34 (Andoniadou et al. 2007; Camper 2011; Carreno, et al. 2017; Chen et al. 2009; Chen et al. 2005; Fauquier et al. 2008; Gaston-Massuet et al. 2011; Gleiberman et al. 2008; Lepore et al. 2005; Mertens et al. 2015), we demonstrated that pituispheres of FAE rats express various stem cell markers. We also showed that cells derived from pituispheres of FAE rats have the ability to migrate, form colonies and develop tumors in immmunodeficient mice. Several studies have shown that tumor recurrence, tumor invasion and tumor resistance to chemotherapy are associated with the high proliferation rate of the tumor stem cells or tumor progenitor cells population (Dai, et al. 2017; Min, et al. 2015; Pastrana, et al. 2011; Smart, et al. 2013). Cancer stem cells,

a small subset of cells within a tumor, have the ability to self-renew and are suggested to promote tumor initiation and progression and have been implicated in driving a more aggressive disease state (Chen, et al. 2012; Hanahan and Coussens 2012; Hanahan and Weinberg 2000, 2011). We show here that pituitary cells of AF rats had the ability to self-renew and develop into solid tumors when transplanted in NOD/SCID mice. Therefore, these cells represent highly proliferative tumor progenitor cells. Together these data suggest that embryonic alcohol exposure programs the pituitary stem cells to develop invasive and aggressive pituitary adenoma under the influence of estrogen.

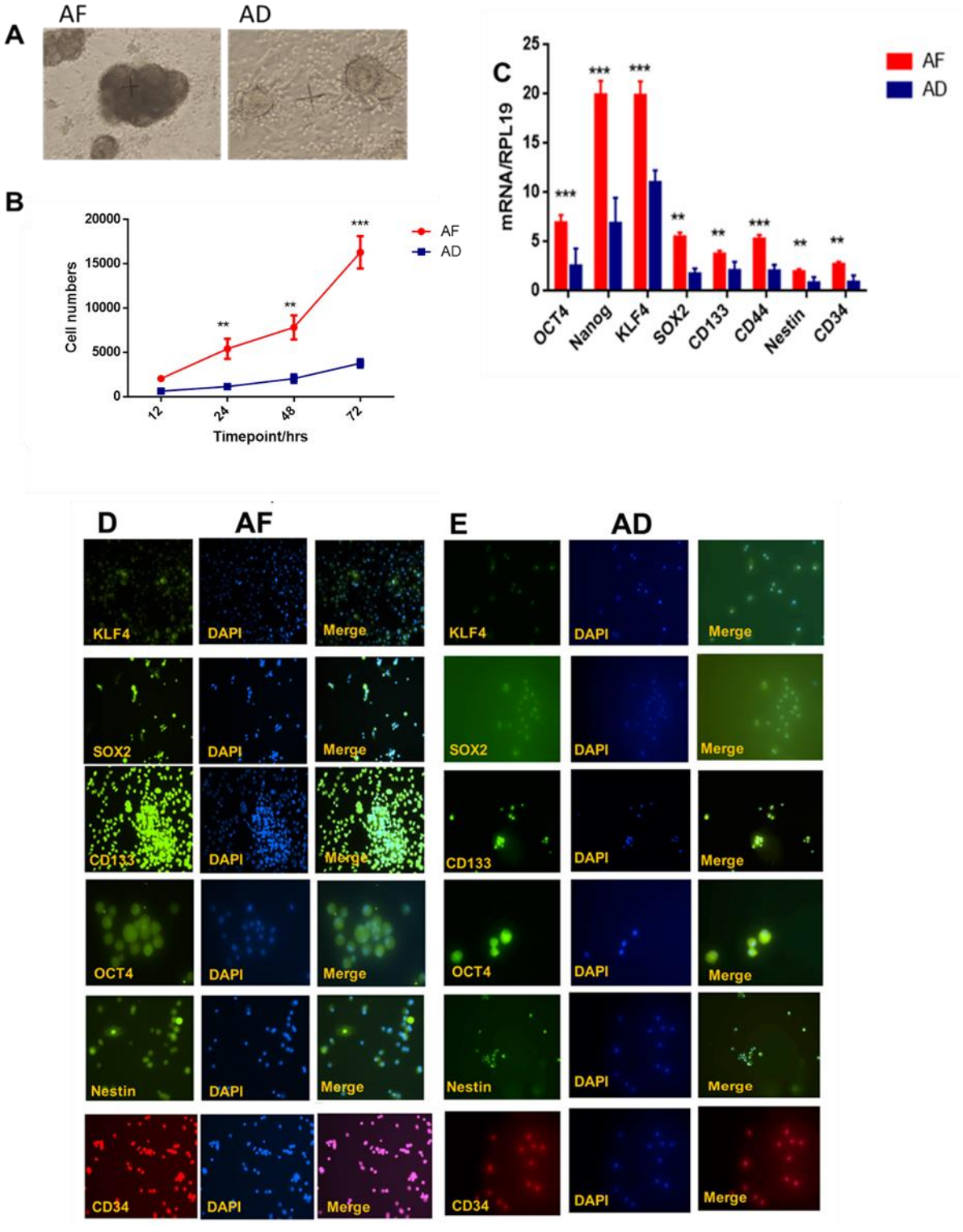
A
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Figure1: Characterization of pituispheres generated from the anterior pituitary of fetal alcohol exposed (AF) or control (AD) rats. Pituispheres were obtained from AF and AD treated rats and maintained in cultures with growth factors. (A) Images of pituispheres from AF and AD rats that were formed after 7 days in cultures in ultralow attachment plates in serum-free growth medium. (B) Brdu cell proliferation assay. Pituitary cells derived from AF and AD pituispheres were cultured in serum free media 2500 cells/well in 96 well plates up to 72hrs. The cell number in the figure refers to the cells positive for Brdu incorporation. Data were analyzed by two-way ANOVA (treatment × time). A significant time × treatment effect on cells proliferation was noted for AF cells (**$P<0.001$), and post hoc comparisons shows AF cells show more proliferation at the 24, 48 and 72 hrs time points (C) Expression levels of various stem cell marker genes (OCT4, Nanog, KLF4, SOX2, CD133, CD44, Nestin and CD34) in pituispheres of AF and AD rats for each gene, expression levels were compared between AD and AF cells by Student's t test (**$P<0.01$, ***$P<0.001$). (D) and (E) Representative photographs of immunofluorescent staining of stem cell-associated transcription factor proteins in AF (D) and AD (E) pituispheres. The magnification shown is 10X.

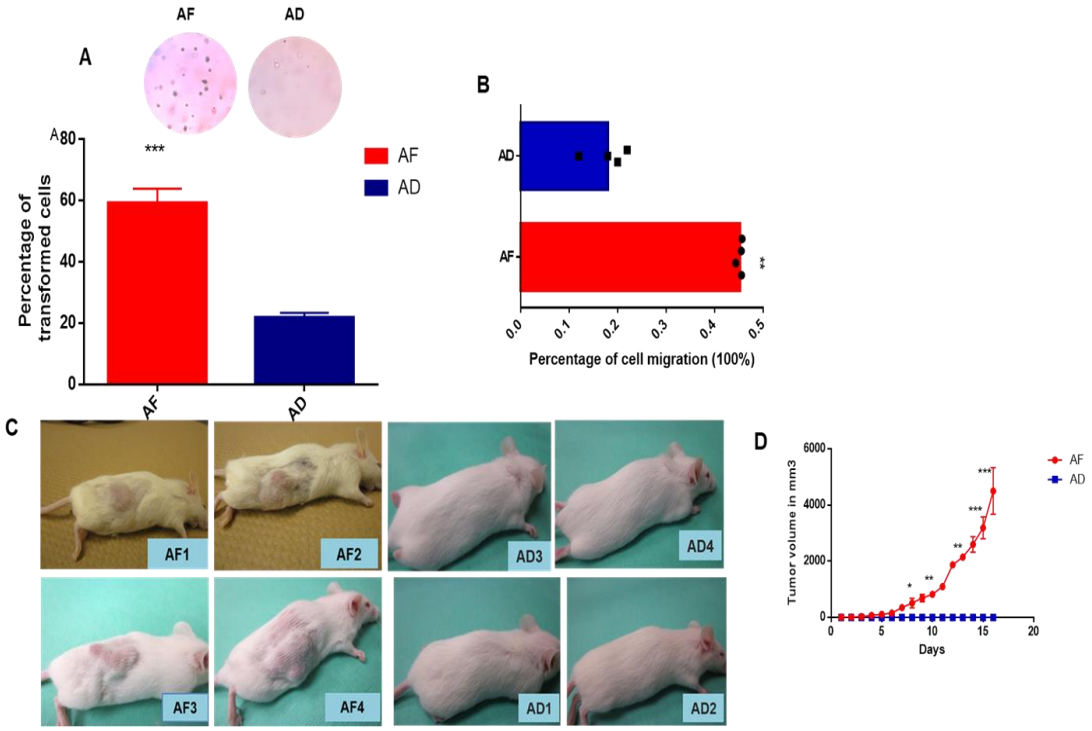

Figure 2: Determination of cell colony formation, migration and tumorigenic ability of pituispheres of fetal alcohol exposed rats (AF) and control rats (AD) treated with estradiol for four months. Pituispheres were obtained from individual AF and AD treated rats and maintained in cultures with serum-free growth medium for several generations. (A) Colony formation in soft agar plates of cells derived from AF and AD pituispheres (top) and the number of transformed cells counts (bottom). Data are expressed as mean ± SEM (n=5-6) and were analyzed using unpaired Student's t test. * P<0.05, **P<0.01, ***, p<0.001 between AF and AD. (B) Cell migration rate of pituitary cells of AF and AD. Data are expressed as mean ± SEM (n=5-6) and were analyzed using unpaired Student's t test. **P<0.01 between AF and AD (C) Representative photos of xenograft mice injected with pituitary tumor cells (5 x10^6 in the right flank area) generated from fetal alcohol exposed rats (AF) or from control rats (AD). (D) Changes in tumor volumes

in xenograft mice injected with AF (n=6) and AD pituispheres (n=5). Data were analyzed by two-way ANOVA (treatment × time). A significant time × treatment effect on tumor growth was noted for AF cells (**P<0.001), and post hoc comparisons shows that AF cells show xenograft tumor growth at the 7, 10 and 15 day time points.

Table 1 Antibody information

Antibody	Cat number	Vendor information
Ki67	Abcam-15580	Abcam
P53	Abcam, PAb 240	Abcam
PTTG	PTTG Antibody (H-160)	Santa Cruz
FGF4	SC, 9006	Santa Cruz
MMP9	Ab38898	Abcam
SOX2	MBS462135	MyBiosource
Nanog	ab106465	Abcam
CD44	Ab 5640s	Abcam
KLF4	Anti-KLF4 (ab72543)	Abcam
CD133	anti-CD133 antibody	MyBiosource
TSH	AB976	Millipore
Growth hormone	BAF1566	R and D system
Prolactin	AF1112	R and D system
Aromatase	ab18995	Abcam
ESR1	ab32063	Abcam

Table 2 primer sequences

Primers	Sequences
FGF4 Forward	ACATCGTTATCAACGGCAGC
FGF4 Reverse	GTCTTCTTCCTCTGCTTCGG
PTTG Forward	GTGCCAACATCAACAAACGA
PTTG Reverse	GCATTGAGGAAGGCTGGAAGA
MMP9 Forward	CGGATCCCCCAACCTTTACC
MMP9 Reverse	AGGTCAGAACCGACCCTACA
αESR Forward	TCGGGAATGGCCTTGTTG
αESR Reverse	AGCTGCGGGCGATTGA
SOX2 Forward	AGAACTAGACTCCGGGCGAT
SOX2 Reverse	ACCCAGCAAGAACCCTTTCC
Nanog Forward	TGCATTTGTCTGAGCTGGGTA
Nanog Reverse	TGGTATGGAGTAGGGTGGGT
OCT4 Forward	GGGGACATCTTGGGTTGGAG
OCT4 Reverse	AGTAGAGCAGTGGGGGTAGG
KLF4 Forward	TGTGACTATGCAGGCTGTGG

KLF4 Reverse	GTGTGGGTCATGTCCACGAT
Sca1	TGAGGATGGACACTTCTCACAC
Sca1	GAACATTGCAGGACCCCAGA
CD34 Forward	AGGTTAGGCCCGAGTGTTTG
CD34 Reverse	TAAGGGTCTTCACCCAGCCT
Nestin Forward	CTGTGGGTGTCAGTGGTCTC
Nestin Reverse	TTAGAGCACCCACCTCCTGT
S100 Forward	AGCTTCTCTGTCTACCCTCCT
S100 Reverse	TCTTCGTCCAGCGTCTCCAT
Pit-1 Forward	CTGTGGTAGCCATGTGTGGT
Pit-1 Reverse	TATTCACATATATGATGGCCTCTCT
SF-1 Forward	CCACCACCGTCTCTCATGTC
SF-1 Reverse	AGGCGTACTTCCCAGGTACT
CD44 Forward	CTACCCCTGAAACACCACCC
CD44 Reverse	TTAGCGCCGCTCTTAGTGCT
CD133 Forward	ACCAAGGAGGTCGCCATCTA
CD133 Reverse	CGAGTCCTTGTCTGCTGGTT

GH Forward	GGCATTTGCCACCTCCTTTG
GH Reverse	GGCATTTGCCACCTCCTTTG
PRL Forward	ACCGTGTGGTCATGCTTTCT
PRL Reverse	AGCCGCTTGTTTTGTTCCTC
TSH Forward	GGAGCATATGGTGAGGACAGG
TSH Reverse	TGGCTCCGTATAGCCACTCA
CYP19A1 Forward	ACTCTACCCACTCAAGGGCA
CYP19A1 Reverse	AGTAGTTTGGCTGTGGCTCC

Conclusions and Future Directions

It is widely accepted that exposure to adverse environmental conditions and lifestyle choices during pregnancy can result in fetal programming that underlies disease susceptibility in adulthood. Fetal alcohol-exposed offspring display many behavioral and physiological abnormalities including neuroendocrine-immune functions, which often persist into their adult life. Previous research conducted in our lab supports the hypothesis that alcohol exposure during gestation leads to increased susceptibility to pituitary tumorigenesis in a rodent model. I found that alcohol exposure during gestation leads to increased plasma estrogen and pituitary aromatase mRNA expression in rodent model of fetal alcohol exposure. Therefore, we tested whether fetal alcohol exposure increases the susceptibility to estrogen-induced pituitary prolactin-secreting tumors (prolactinomas), a commonly occurring pituitary tumor in humans.

A review of the literature on the effect of adult alcohol exposure and in utero alcohol exposure provided evidence for increased incidence of prolactinoma development. Some of the clinical cases have found chronic or binge alcohol exposure elevated plasma prolactin level. Animal model studies also showed that both acute and chronic alcohol-exposure increased plasma prolactin and pituitary proliferation labeling index. However, there are limited studies conducted to determine the effect of fetal alcohol exposure on the pituitary tumor development, in particular pituitary prolactinoma development.

In Chapter 2 I showed that fetal alcohol exposed animals had increased levels of pituitary weight, pituitary prolactin (PRL), plasma PRL, pituitary aromatase, and plasma estrogen. I also showed that estradiol treatment time-dependently increased pituitary

weight more in fetal alcohol exposed animals than in control animals. After 120 days of estradiol treatment, I found both fetal alcohol exposed rats and control rats developed large pituitary tumors, but pituitary tumors in fetal alcohol exposed rats are hemorrhagic and showed expansion to the surrounding tissue. Pituitary tumor from alcohol exposed animals also showed marked cell proliferation and elevated expression of genes and proteins related hemorrhage (PTTG, FGF4 and MMP-9), and multipotency (SOX2, Oct4 and CD133). These data suggest that fetal alcohol exposure may promote development of aggressive tumors.

Chapter 3 summarized the evidence of existence of stem cells in pituitary tumors of alcohol-exposed animals. This evidence came from the observation that pituispheres obtained from fetal alcohol exposed rats expressed elevated levels of multipotency markers (OCT4, NANOG, KLF4, SOX2, CD133, CD44, nestin and CD34) and showed higher cell proliferation, migration and colony formation rates and developed tumors in immune-compromised mice. These data provide evidence that fetal alcohol exposure rats potentially develop larger and possibly aggressive pituitary tumor.

Future studies should focus on the role of epigenetic mechanisms involved in alcohol-specific expression of genes related to Wnt signaling and stemness (possibly by using CHIP). An understanding of the mechanisms involved in increased pituitary tumorigenesis in fetal alcohol-exposed animals would ultimately lead to better prevention and treatment strategies for aggressive pituitary tumors.

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