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The Characterization of the Neuropathological Consequences of Plac1 Ablation in a Mutant Mouse Model

Jacob Jacob Jacob
University of South Florida, bourgeois.jake@gmail.com

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The Characterization of the Neuropathological Consequences of *Plac1* Ablation in a Mutant Mouse Model

by

Jacob R. Bourgeois

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Public Health with a concentration in Toxicology and Risk Assessment
Department of Environmental and Occupational Health
College of Public Health
University of South Florida

Co-Major Professor: Giffe Johnson, Ph.D.
Co-Major Professor: Raymond D, Harbison, Ph.D.
Michael E. Fant, MD, Ph.D.

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ABSTRACT

Placenta-specific 1 (Plac1) is an X-linked gene that is essential for normal placental development. Previous studies have shown that Plac1 is also expressed in the fetal brain and paternally imprinted. Its expression in the fetal brain is markedly downregulated immediately after birth. Plac1 ablation predisposes Plac1-null males and X<sup>m</sup>-X Hets (inactive maternal allele) to an increased risk of developing lethal postnatal hydrocephalus suggesting a functional role for Plac1 during embryonic development. The objective of this study was to characterize the effect of Plac1 on brain development and postnatal function. In order to address this, a mutant Plac1 mouse model, established on the C57BL/6J background, was studied. Formalin-fixed, paraffin-embedded whole mount embryos and brain sections were obtained at various stages of development. Plac1 expression was assessed by qRT-PCR and immunohistochemistry (IHC). Brain structure was assessed by histopathological and magnetic resonance imaging (MRI) analysis. Behavioral analysis was conducted using the PhenoMaster automated cage system and a battery of classical behavioral tests. Our results revealed Plac1 expression throughout the embryonic brain when assessed by qRT-PCR and IHC at E16.5 and E18.5. MRI analysis of an adult Plac1 knockout (KO) brain revealed microcephaly (14%), reduced ventricular volume, and increased heterogeneity of the medulla compared to a WT brain. Consistent with these findings, H&E staining of the KO brain revealed a smaller cortical mantle, a dysmorphic hippocampus, and a dysmorphic cerebellum with reduced folia. IHC analyses of NF-M, NeuN, and Iba1 immunostaining revealed significant reductions in axonal and neuronal development and
increased activated microglia in KO brain, but not in X<sup>m</sup>-X Hets. Although no structural abnormalities were detected in X<sup>m</sup>-X Hets, behavioral analyses did reveal reduced activity and behaviors consistent with increased anxiety. In conclusion, Plac1 is a paternally imprinted, X-linked gene that is associated with abnormal brain development, reduced activity, and specific behavioral abnormalities.
CHAPTER ONE:
INTRODUCTION

The *PLAC1*/*Plac1* Gene

Placenta-specific 1 (*PLAC1*) is an X-linked gene that was originally believed to be expressed solely in the placenta. Northern blot analysis and *in situ* hybridization at the time suggested *PLAC1* expression was restricted to placental trophoblasts during development (Cocchia et al., 2000; Fant et al., 2002). Expression of murine *Plac1* is highest in the placenta from embryonic days 7.5-14.5 (E7.5-E14.5). Expression was reported to be restricted to trophoblast-derived cells, including cells in the spongiotrophoblast, placental labyrinth, and trophoblast giant cells. In humans, *PLAC1* expression remains relatively constant from 22 to 40 weeks of development (Fant et al., 2002; Massabbal et al., 2005). Recent research has elucidated a more complicated expression profile where its expression was shown to occur throughout the entire embryo, in particular, the brain, heart, kidney, and lungs (Jackman, Kong, & Fant, 2012; Kong, Jackman, & Fant, 2013). Interestingly, expression has also been detected in a wide range of tumors and transformed cell lines (J. Chen et al., 2006; Koslowski et al., 2007; Silva et al., 2007). Placental tumor choriocarcinoma cell lines BeWo, JAR, and JEG, neuroblastoma cell line LA1-56, and a number of others had significant *PLAC1* expression. *PLAC1* was also expressed in approximately 82% of primary breast tumors and 50% of gastric tumors.

*PLAC1* maps to Xq26, 65 kilobases telomeric to the gene hypoxanthine-guanine phosphoribosyltransferase, which codes a protein important in purine metabolism (Cocchia et al.,
This area of the X chromosome is considered important for fetal development. Large deletions (200-700 kb) around the \textit{Hprt} locus in several mice resulted in a runty phenotype or death (Kushi et al., 1998). Also, this region has been implicated in interspecific hybrid viability (Zechner et al., 1996). The deletion of the \textit{Hprt} gene itself does not result in abnormal placental phenotype in mice, suggesting that some gene(s) nearby, such as \textit{PLAC1}, may be responsible (Searle, Edwards, & Hall, 1994).

\textit{PLAC1} has six exons, with the last exon coding for the protein (Y. Chen, Moradin, Schlessinger, & Nagaraja, 2011). \textit{PLAC1} contains two active promoters, P1 and P2. P1 lies upstream of the first exon, while P2 lies just upstream of exon 4. This unique structure is conserved in mouse \textit{Plac1}. Nuclear receptors retinoid X receptor alpha (RXR\textalpha) and liver X receptor (LXR) activate both promoters, and putative RXR\textalpha binding sites have been detected on P1. Both receptors are expressed prominently in the placenta during development. RXR\textalpha is the dominant isoform in placenta, and selective knockout is associated with several severe placental defects and potential embryo lethality (Sapin, Dolle, Hindelang, Kastner, & Chambon, 1997). In normal placenta, P2 is the primary promoter. However, in studied cancerous cell lines, the preferred promoter is variable (Y. Chen et al., 2011). The significance of the different PLAC1 isoforms is unknown.

\textbf{The PLAC1/Plac1 Protein}

The putative PLAC1 protein is relatively small. The human open reading frame (ORF) encodes a protein of 212 amino acids; the mouse Plac1 protein is 173 amino acids but very homologous to human – 75\% similar at the DNA level and 60\% at the amino acid level (Cocchia et al., 2000). The amino acid sequence contains a signal peptide, suggesting that PLAC1 is likely
targeted to the secretory pathway and exists as an extracellular protein. Interestingly, both proteins share sequence homology with zona pellucida 3 protein, a specific sperm-binding protein in the zona pellucida critical for fertilization. This zona pellucida domain (ZPD) has been found and characterized in other secreted glycoproteins, such as uromodulin and betaglycan (Bork & Sander, 1992; Jovine, Qi, Williams, Litscher, & Wassarman, 2002). In particular, this domain is used for cross-linking and polymerization along the sequence, suggesting that PLAC1 may also possess such an attribute. The presence of the ZPD, alongside immunohistochemical localization studies, suggest that PLAC1 is a secreted or membrane-bound protein. Furthermore, the lack of cytoplasmic signaling domains in PLAC1 suggests that the protein likely modulates signaling transduction pathways mediated by other receptors (Fant, Farina, Nagaraja, & Schlessinger, 2010).

PLAC1 also appears to elicit an antibody response. Some patients with high-PLAC1 expressing cancers were found to have developed circulating anti-PLAC1 antibodies. In a study of patients with colorectal cancer, approximately 50% of tumor samples expressed detectable PLAC1 mRNA (Liu et al., 2008). Of patients with these tumors, more than half expressed responsive T cells, and about 30% expressed spontaneous auto-antibodies against PLAC1. In a study of patients with hepatocellular cancer, 32% of samples expressed PLAC1, and 4% of all patients were seropositive for PLAC1 antibodies (Dong et al., 2008). Preliminary analysis of the patients of both studies suggested that the presence of these PLAC1 auto-antibodies provided a significant survival advantage. These data suggest not only that PLAC1 has some distinctive role in cancer biology, but also that targeting the PLAC1 antigen may be a viable therapeutic strategy. Indeed, PLAC1 silencing and PLAC1 neutralization by antibodies in MCF-7 breast
cancer cells resulted in decreased motility, invasiveness, and cell proliferation (Koslowski et al., 2007).

**PLAC1 Function**

The mechanism of action for PLAC1 is unknown. The prior discussion on the putative sequence of the PLAC1 protein and experiments exploring the relationship of PLAC1 in various cancers suggests that the protein plays some role in migration and invasion of placental processes. The interactive ZPD and lack of cytoplasmic signaling domains suggests modulation of other signaling protein pathways. Differential microarray analysis of E18.5 KO placentae revealed downregulation of several genes important in embryogenesis, including Bmp, Wnt, and Pdgf (Fant, unpublished data). Canonical pathways implicated include “ES Cell Pluripotency”, “Integrin Signaling”, “Glioblastoma Multiform Signaling”, and “Axonal Guidance Signaling”.

PLAC1 is required for normal placental development. Intensive study of a mutant mouse model in our laboratory demonstrated that ablation of Plac1 resulted in significant placentomegaly (100% increase) and intrauterine growth retardation (IUGR) (Jackman et al., 2012). The effect was most pronounced in male Plac1 knockouts (KOs), followed by X<sup>m</sup>-X Hets (inactive maternal allele). No female KOs were identified postnatally. While examining the postnatal phenotype of mutant Plac1 mice, a number of interesting and important observations were made. First, KO males exhibited decreased postnatal viability. Approximately 20-25% of the expected number of KO males survived. Second, and more surprisingly, although some surviving KO males appeared normal and were fertile, 22% developed lethal hydrocephalus (HC) at 4-8 weeks of life (Kong et al., 2013). Third, although the X<sup>m</sup>-X heterozygotes exhibited normal postnatal viability they also exhibited an increased risk (11%) of developing lethal
hydrocephalus at 4-8 weeks of age. These observations strongly suggested a functional role for brain-derived Plac1 in brain development and function.

In the work described in this thesis, we further examined Plac1 expression in the brain during development. We show that Plac1 KO mice exhibit a number of histological abnormalities and structural malformations. Furthermore, we report that female Plac1 Hets (X<sup>m-</sup> X) exhibit reduced activity likely related to anxiety using both automated cage observation and specific behavioral testing.
CHAPTER TWO:
METHODS

**Plac1 Mutant Mouse Model**

Mutant mice were bred against a C57/BL6 background as described previously (Jackman et al., 2012). The Plac1 open reading frame on exon 6 was deleted in murine embryonic stem (ES) cells by the Knockout Mouse Project (KOMP) VelociGene approach (KOMP-NIH initiative; [http://www.velocigene.com/komp/detail/10766](http://www.velocigene.com/komp/detail/10766)). Blastocysts were injected with Plac1-null ES cells to produce chimeras (KOMP). Mice were bred against C57BL/6 background once germline transmission was established. Procedures and protocols such as timed mice matings were carried out in accordance with Institutional Animal Care and Use Committee (IACUC) of the University of South Florida College of Medicine Protocol Number R4228 (Appendix A).

**Genotyping Determination for Mice**

Genotyping was performed using PCR as described elsewhere (Kong et al., 2013). DNA was isolated from embryonic mouse tails using a DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA). Plac1 genotype was determined by PCR, using the following primers: 50-CCAATCATGTTCACCCACATTTCTAC (WT forward);

50-CCCTAAAAGAGCTATCATGGCATCT (reverse);

50-GCAGCCTCTGTTCCACATACACTTCA (neomycin universal forward). The cycling parameters were: 94°C for 5 min, followed by 10 cycles of 94°C for 15 sec, 65°C for 30 sec
(decreased by 1°C at each repeat), and 72°C for 40 sec; followed by 30 cycles of 94°C for 15 sec, 55°C for 30 sec, and 72°C for 40 sec. PCR products were terminated with a final extension at 72°C for 5 min, then held at 4°C. A 1% agarose gel was used to visualize the generated wildtype and mutant bands at 548 and 326 bp, respectively.

**Quantitative Measurement of Brain Plac1 mRNA**

WT pregnant females were euthanized at E16.5 and E18.5 during gestation and the brains collected. Specific regions of each embryo’s brain were dissected under a dissecting microscope, placed in RNAlater (Qiagen, Valencia, CA), and pooled with the same region collected from the other littermates. Total RNA was then prepared using the AllPrep DNA/RNA Kit (Qiagen). Plac1 mRNA was measured by qRT-PCR and normalized against 18S ribosomal RNA as described in Kong *et al.* 2013 (*Kong et al.*, 2013). The brain regions (cerebellum, medulla, pons, etc.) pooled from embryos derived from one litter constituted 1 sample. The average expression for each region was determined from at least 2 litters.

**Hemotoxylin and Eosin (H&E) Staining**

H&E staining was performed by the Moffitt Tissue Core ([http://moffitt.org/research--clinical-trials/research-cores--technology/tissue-core](http://moffitt.org/research--clinical-trials/research-cores--technology/tissue-core)) using a standard protocol.

**MRI Analysis**

Mice underwent a T2-weighted MRI. Regions of interest, in particular the ventricles, medulla, and hippocampus, were drawn and compared using the Aedes software package (Aedes, Finland, [http://aedes.uef.fi/](http://aedes.uef.fi/)).
**Immunohistochemistry**

Paraffin embedded tissue were cut into 5 uM sections onto glass slides. The slides were deparaffinized in xylene (Thermo Fisher Scientific, Waltham, MA) for 2x10 min, rehydrated in 100% EtOH for 2x5 min, 95% EtOH for 5 min, 75% EtOH for 5 min, 50% EtOH for 5 min, and finally in dH₂O for 5 min. The slides were washed in Phosphate buffered saline (PBS) for 10 min. and excess liquid drained. The slides were washed with PBT (PBS + 0.1% Tween-20) for 3x5 min, and then incubated in blocking buffer (10% serum of secondary antibody host serum in PBT) for 1 hour at room temperature. The slides were then incubated in primary antibody in blocking buffer overnight at 4°C. Non-specific staining was determined by incubating with homologous non-immune serum (dilution equivalent to primary antibody). The slides were then washed in PBT 3x5 min. and incubated with IgG from primary antibody host (VectaStain Elite ABC kit, Vector Labs, Burlingham, CA) for 1 hour. The slides were placed in ABC reagent (per protocol) for 30 min and washed in PBT 3x5min, stained using dianinobenzidine substrate, then mounted. Primary antibodies and dilutions used include anti-Plac1 (Abbomax, San Jose, CA, 1:200 dilution), anti-NF-M (Santa Cruz Biotechnology, Dallas, TX, 1:50 dilution), anti-NeuN (Millipore, Billerica, MA, 1:125 dilution), and anti-Iba1 (Wako Pure Chemical Industries, Osaka, Japan, 1:333 dilution).

**Image Analysis**

Immunohistochemical pictures were quantitatively analyzed using ImageJ v1.46r software (NIH, http://imagej.nih.gov/ij/download.html). Color threshold RGB values were obtained manually, then applied systematically to each sample using built-in function “Analyze
Particles” to obtain area percent stain or number of cell bodies. Parameters were chosen appropriately to accurately capture cell bodies or positive staining regions.

**PhenoMaster Analysis**

The PhenoMaster system (TSE Systems GmbH, Bad Homburg, Germany) is a modular automated high-throughput home-cage environment capable of recording physiological and behavioral data with high spatial and temporal resolution (Clemens, Jansson, Portal, Riess, & Nguyen, 2014; Urbach et al., 2014). The setup used for these studies allowed simultaneous observation of 12 mice. The experimental window consisted of 5 days. The 5 day observation period was split into two time frames – the first 24 hours of monitoring, or “Day 1”, and the remaining 96 hours, or “After Day 1”. This was done to separate the acclimation or exploration period from the period of normal activity. There was further stratification by light, dark, and both cycles. Raw data was compressed within the PhenoMaster software. Data was extracted and analyzed using Microsoft Excel.

**Specific Behavioral Testing**

Mice were also subjected to a battery of well-described rodent behavioral tests, including the Elevated Plus Maze (EPM), Open Field Test (OF), Y-maze, Novel Object Recognition Test (NOR), Rotarod, and Radial Arm Water Maze (RAWM).

**Elevated Plus Maze**

The EPM was employed to evaluate anxiety as described elsewhere (Arendash et al., 2001). The maze consisted of four arms (30 x 5cm) connected by a central area (5 x 5cm), all
constructed of plywood and painted black. Two opposite-facing arms were open, while the other two were enclosed by aluminum sheet walls 15 cm high. The maze was elevated 82 cm above floor level. One mice at a time was placed in the central area, and its movement was recorded by ceiling video camera during a single 5 minute trial. The data was analyzed using video tracking software ANY-maze (ANY-maze, Stoelting, IL). Between mice, the maze was cleaned using 70% ethanol to minimize olfactory cues.

**Open Field Test**

Animals were monitored for 15 minutes in a 40 cm square open field with video tracking software (ANY-maze, Stoelting, IL), under moderate lighting as a standard test of general activity.

**Y-maze**

Each animal was placed in a walled Y-maze and allowed to roam freely for a single 5 minute trial. The sequence of arm entries and total number of arm choices were recorded. Spontaneous alternation, or the proportion of arm selections made consecutively without repetition, was calculated and expressed as a percentage (Anisman, 1975).

**Novel Object Recognition Test**

Described elsewhere (Brownlow et al., 2014). The test consists of a 40 x 40 cm area monitored by video tracking (ANY-maze, Stoelting, IL). Two objects similar in scale to the mouse were placed along the center line of the arena approximately 3-5 cm from the outside wall. Each animal was given three acclimation trials of 5 minutes each with 5 minutes in
between. After each trial, the arena and object cues were cleaned with 70% ethanol to minimize olfactory cues. Five minutes after the final acclimation trial, one of the objects was replaced by a novel object. The mice were then observed by video tracking over a 5 minute exploratory trial. Working memory was assessed by the exploration index, defined as the time spent exploring the novel object over total exploration time.

**Rotarod**

Described elsewhere (Morgan et al., 2008). Mice were placed onto the round portion of a motorized circular rod (Ugo Basile Rota-rod model 7750). Mice were required to walk at the speed of rod rotation to prevent falling. The rod slowly accelerated every 30 seconds from 2.5 RPM to 34 RPM over 5 minutes. Time until falling was recorded for each mouse. Mice were given three trials each day, separated by at least ten minutes, for 5 consecutive days. The average time until falling for each day was recorded. The maximum possible time on the rod is 300 seconds, which is rarely met.

**Radial Arm Water Maze**

The test contained six swim arms radiating from an open central area, with a submerged hidden escape platform located at the end of one of the arms. Starting with a randomly selected arm, the mice were placed at the end of an arm and was allowed to swim in the maze for up to 60 seconds to find the escape platform. The escape platform was located in the same arm each trial. On day one, mice were given 15 trials (three per block) alternating between a visible platform (above the water) and a hidden one (below the water). The following day, they were given 15 trials with every trial using a hidden platform. The start arm was varied for each trial to
emphasize mice learning by spatial cues rather than motor learning. Entry of all four limbs in an incorrect arm or failure to enter the correct arm within 20 seconds was scored as an error. Errors for the three trial blocks were averaged for data analysis. On the third day, a reversal experiment was performed with the goal placed in the arm opposite (180°) from the original location. Mice were given 15 trials to unlearn the first location and find the second location through training. On the fourth day, the arms were removed, the platform was raised above the water, and a flag was attached to the platform to confirm all mice were capable of seeing and climbing onto the platform (open pool). The time taken to reach the platform was recorded.

**Statistical Analysis**

Data obtained from ImageJ stain analyses were compared using Student’s T test. Variables obtained from PhenoMaster analysis were stratified by age, experimental phase, and day status (ie. light, dark), and compared using Student’s T-test. Variables recorded from the remaining specific behavioral tests were compared using Student’s T-test. Comparison of variables along time were analyzed using repeated measures ANOVA. An alpha of 0.05 was applied for all tests performed.
CHAPTER THREE:

RESULTS

PLAC1 is Expressed Throughout the Fetal Brain

In order to establish the pattern of Plac1 expression during fetal development, Plac1 mRNA was measured in various regions of the brain using quantitative real-time PCR (qRT-PCR). Brains were obtained from WT embryos at E16.5 and E18.5 (n = 2 litters) and divided into distinct anatomical regions. Total RNA was subsequently obtained and quantified. Plac1 mRNA was detected throughout the fetal brain especially the pons, midbrain, cerebellum, and hippocampus (Figure 1). These findings are consistent with previously reported data from our laboratory that localized Plac1 mRNA to the periventricular cortex, pons/medulla, and cerebellum by in situ hybridization (Kong et al., 2013).

Figure 1. Plac1 mRNA expression by anatomy in the mice brain at E16.5 and E18.5. n = 2 each. Plac1 mRNA was obtained from respective areas of mice brain at appropriate developmental stage. Individual samples were done in triplicate.
Localization of *Plac1* expression during development was further explored using immunohistochemistry (IHC). Anti-Plac1 polyclonal antibodies were used to perform DAB-brown immunostaining on whole-mount WT, X<sup>m</sup>-X Hets, and KO embryos at E18.5 according to protocol (Figure 2). In the WT embryo, Plac1 was detected throughout the brain, particularly in the vomeronasal organ, the olfactory bulb, and the cerebral cortex. Plac1 expression in the Het and KO were appreciably lower, verifying the specificity of the anti-Plac1 antibody used. These findings are consistent with the qRT-PCR data, suggesting that *Plac1* may have a general role in brain development.

**Figure 2.** *Plac1* localization by Immunohistochemistry using anti-Plac1 antibody of E18.5 whole-mount embryos. OB – olfactory bulb, VM – vomeronasal organ, C – cerebral cortex.
Ablation of *Plac1* Results in Abnormal Brain Structure

In order to gain insight into possible neurological functions for *Plac1* during brain development, brains of postnatal mice of each genotype were obtained and compared using a variety of relevant criteria. H&E staining of WT and KO mice revealed a number of striking histological changes, including decreased size of lateral ventricles, disorganized neuronal distribution, especially on the parietal cortex, and a simpler cerebellum with reduced folia (Figure 3). These findings are consistent with prior MRI analysis of WT and KO brains, which showed KO brains have 12% decreased overall brain volume and 14% decrease in ventricular volume, adjusted for total brain volume (Figure 4). Furthermore, MRI revealed greater tissue heterogeneity in the KO medulla compared to WT, indicating possible structural abnormalities in the observed brain (See Tables 1 and 2).

**Figure 3.** H&E stain comparison of KO and WT mice brains. Significant alterations include narrowed ventricles (A vs. D), abnormal cell organization with disorganized parietal cortex layering (B vs. E), and truncated cerebellar folia (C vs. F).
**Figure 4.** MRI visualization of KO and WT brains. KO brain (left, red) is visibly smaller than WT brain (right, green). MRI image analysis results displayed in Tables 1 and 2.

<table>
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<tr>
<th>Region of Interest</th>
<th>Relative Volume</th>
<th>Relative Normalized Volume</th>
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<tr>
<td>Lateral Ventricles</td>
<td>0.8340898</td>
<td>0.962676</td>
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<tr>
<td>Third Ventricle</td>
<td>0.5471264</td>
<td>0.631473</td>
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<td>Fourth Ventricle</td>
<td>0.4681529</td>
<td>0.540325</td>
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<tr>
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<td><strong>0.861398</strong></td>
</tr>
<tr>
<td>Brain</td>
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<td>1</td>
</tr>
</tbody>
</table>

**Table 1.** MRI volumetric comparison of KO and WT brains. The KO brain is approximately 14% smaller than WT control. Furthermore, ventricular volume normalized to total brain smaller is 14% smaller in KO than WT control.
<table>
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<th>Relative Mean CSF Intensity</th>
<th>Relative St. Dev</th>
<th>Difference?</th>
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<td>0.9889617</td>
<td>Very similar</td>
</tr>
<tr>
<td>Third Ventricle</td>
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<td>0.91115869</td>
<td>similar</td>
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<tr>
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<td>Different STD</td>
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</tbody>
</table>

Table 2. MRI voxel intensity comparison of KO and WT brains. The standard deviation of voxel intensity in the medulla is approximately 38% greater in the KO than WT control, indicating significant tissue heterogeneity.

A *Plac1* KO Mouse Exhibited Altered Expression of NeuN, NF-M, and Iba1, While X<sup>em</sup>X Hets Appeared Unaffected

In order to characterize specific neurological disturbances *Plac1* ablation may instigate later in life, mouse brains of each genotype were obtained and compared using immunohistochemistry of various markers. Specifically, anti-Neuronal Nuclei (NeuN) and anti-Neurofilament-M (NF-M) were employed to assess potential neuronal and axonal damage. A KO male brain exhibited significantly less NF-M expression than a male WT control, particularly in the corpus callosum and caudate putamen (Figure 5). Analysis of anti-NeuN staining revealed decreased staining and neuronal cell count in the cerebellum, hippocampus, and cortex (Figure 6). These data revealed that ablation of *Plac1* expression resulted in decreased expression of NeuN and NF-M, and interfered with normal axonal guidance.

Furthermore, immunohistochemical staining against Iba1, a marker for activated microglia, allowed assessment of potential inflammatory consequences of *Plac1* ablation in the postnatal nervous system. Anti-Iba1 staining revealed significantly more activated microglia throughout the brain in KO and Hets compared to WT equivalent later in life (Figure 7).
Figure 5. Comparison of NF-M expression between male WT (WT-M) and male Plac1 KO mice. KO mice brain show reduced NF-M expression and decreased axonal migration. NF-M reduction in the KO brain compared to WT-M is evident throughout the entire brain, especially in the corpus callosum, caudate putamen, and pons-medulla (A-C). Axonal migration in the caudate putamen is visually hindered in the KO brain (D-F).

Figure 6. Comparison of NeuN expression between male WT (WT-M) and male Plac1 KO mice. WT-M neuronal expression in the cortex, cerebellum, and hippocampus (A-C) visually outweighs expression in the KO equivalent (D-F).
Figure 7. Iba1 IHC comparison between male WT (WT-M) and male Plac1 KO mice. KO mice brain show significantly more activated microglia than WT. Pattern exists throughout the brain, but particularly in the cortex.

Chronically activated microglia are usually suggestive of neuronal damage and neurodegenerative disorders (Dheen, Kaur, & Ling, 2007). Plac1 ablation thus may predispose mice to increased neuronal loss later in life.

Interestingly, NeuN, NF-M, and Iba1 expression of Xm-X Hets seem largely unchanged compared to female age-matched WT mice. At 11 months, there is no appreciable difference between stains for any marker (Figure 8). This similarity is confirmed using quantitative analysis done through ImageJ. It appears that the paternal allele has enough function to rescue Xm-X Hets from histological neuronal and axonal abnormalities throughout adulthood.

Reduction of Plac1 Expression Affects Various Behavioral Parameters in Mice

In order to detect potential downstream behavioral and phenotypical changes associated with reduction Plac1 during development, female WT (WT-F) and Xm-X Hets (HETs) at various matched ages were analyzed in both automated cage environments and classical test apparatuses. Mice were first continuously monitored using the TSE PhenoMaster system.
Figure 8. Comparison of staining of female WT (WT-F) and Plac1 X^m-X heterozygotes (HET) at 11 months. WT-F and HETs stain similarly using NF-M (A vs. B). NeuN is similar at the cerebellum (C vs. D), cortex (E vs. F), and hippocampus (G vs. H). Iba1 is similar throughout the brain (I vs. J). Quantitative threshold analysis using ImageJ (n = 3 each) confirms visual similarities (K-M).
**PhenoMaster**

PhenoMaster analysis revealed a number of significant differences in activity and mobility parameters between WT-F and HETs (Figure 9). During Day 1, HETs at 5 months ($n_{WT-F} = 4$, $n_{HET} = 4$) exhibit decreased fine beam breaks ($p = 0.006$), increased locomotive speed ($p = 0.038$), and increased time asleep in the dark phase ($p = 0.014$), and decreased time active considering both phases ($p = 0.049$). Over the entire experimental period, HETs at 5 months show noticeably increased rearing activity ($p = 0.049$). HETs at 12 months ($n_{WT-F} = 7$, $n_{HET} = 4$) spend significantly more time in the periphery of the cage rather than the center in the light phase of both Day 1 ($p = 0.037$) and After Day 1 ($p = 0.039$), and increased rearing during the dark phase after Day 1 ($p = 0.050$). Interestingly, when observing the sleeping habits of HETs at 5 months, these mice slept much more during the Day 1 than After Day 1, when the opposite is expected. There were no significant alterations in feed and drink habits, or metabolic parameters along either experimental period or age group. Collectively, these findings show that reduced $Plac1$ expression during development is associated chiefly with decreased overall activity during the acclimation phase at 5 months, and increased exploration and rearings throughout the entire experiment at 12 months.

**Specific Behavioral Testing**

WT-F and HETs were further analyzed using a variety of classical behavioral tests. In order to assess mobility and behaviors associated with anxiety, the Elevated Plus Maze (EPM) and Open Field (OF) test were employed. The Y-maze was used to assess spatial working memory. The Novel Object Recognition (NOR) test provided a measure of cognition and recognition memory. Motor coordination, balance, and motor learning were assessed using the
Figure 9. PhenoMaster analysis and comparison of female WT (WT-F) and Plac1 X<sup>im</sup>-X heterozygotes (HET) at 5 months (n = 4 each) and 12 months (n<sub>WT-F</sub> = 7, n<sub>HET</sub> = 4). At 12 months, WT-F mice at 12 months spend significantly more time in the center in both the acclimation phase (A) and during the Light phase in After Day 1 (B). HETs at 5 months are asleep more often in the Dark phase of Day 1 (C) and display an abnormal sleep acclimation between experimental phases (D). Rearing were more common in HETs at 5 months during the Light phase overall (E) and HETs at 12 months during the Dark phase After Day 1 (F). Fine movements were significantly reduced in HETs at 5 months during the Dark phase in the acclimation phase (G). HETs at 5 months on Day 1 were also less active overall (H) yet faster during the active phase (I). Data represents means ± SEM (**p < 0.05, ***p < 0.01)
Rotarod. Finally, spatial working memory and spatial learning were compared between the two groups using the Radial Arm Water Maze (RAWM).

The EPM revealed further differences between WT-F ($n_{WT-F} = 5$) and HET ($n_{HET} = 3$) mice at 16 months (Figure 10). There were no appreciable differences between HETs and WT-F mice with regard to most classical anxiety-related parameters, including percent time in open arms ($p = 0.254$), percent time mobile in open arms ($p = 0.277$), and ratio distance traveled in open arms ($p = 0.285$). However, none of the HETs explored the entirety of the open arms, as shown by respective heat maps, and HET mice heads entered the open arm zone less frequently ($p = 0.034$). Furthermore, there were substantial differences between genotypes with regard to locomotor skills, including total distance traveled ($p = 0.001$) and time immobile ($p = 0.007$).

![Figure 10](image)

**Figure 10.** Elevated Plus Maze analysis of female WT (WT-F, 16 mo., $n_{WT-F} = 5$) and *Plac1 X*<sup>m</sup>-X heterozygotes (HETs, 16 mo., $n_{HET} = 3$). HETs traveled significantly less and were much less mobile than WT-F, and HET mice heads entered the open arm zone less frequently (A). HETs traveled less and spent less time than WT-F in the Open Arms, but were not statistically different (B). However, unlike the WT-F, HETs did not explore most of the open arms (C). Heat maps represent group occupancy plot of mice center position. Data represents means ± SEM (**$p < 0.05$, ***$p < 0.01$, ****$p < 0.005$)
Further behavioral testing found no additional significant differences between WT-F and HETs. OF testing (n\textsubscript{WT-F} = 7, n\textsubscript{HET} = 4) found a small but insignificant reduction in total distance traveled, both overall (p = 0.279) and by three minute intervals (p = 0.99). HETs were slightly more immobile (p = 0.103) and slower (p = 0.284), but were identical in regards to propensity to the center (p = 0.941), the chief measurement for anxiety (Figure 11). Y-maze analysis showed no differences in arm entries (p = 0.640) or percent alternation (p = 0.191) between WT-F and HETs (Figure 12ab). The NOR test (n\textsubscript{WT-F} = 7, n\textsubscript{HET} = 3) showed no differences in attention paid to the novel object (p = 0.896) and only slight reduction in discrimination ratio (p = 0.627) (Figure 12cd). Rotarod analysis (Figure 12e) showed no overt deficiencies between WT-F and HETs in continual motor control and learning in day 1 of testing (p = 0.175) nor day 2 (p = 0.278). The RAWM (n\textsubscript{WT-F} = 6, n\textsubscript{HET} = 3) showed a very modest increase in entry errors for HETs (p = 0.884). There was no difference in error improvement over time in neither day 1 (p = 0.860), nor day 2 (p = 0.070). Furthermore, reversal of the maze showed no learning impairment over time (p = 0.573), and open pool testing confirmed no visual or motor impairments (Figure 13).
Figure 11. Open field test comparison of 16 mo. female WT mice (WT-F, n_{WT-F} = 7) and 16 mo. Plac1 X^{m}-X heterozygotes (HETs, n_{HET} = 4). Mice are placed into a novel box environment and resultant activity is recorded. HETs travel only slightly less than WT-F (A), and are comparable over time (B). HETs are slightly less mobile (C) and slightly slower (D) than WT-F, but these observations are not statistically significant. HETs and WT-F are similar with regards to time spent in the center of the field (E).

Figure 12. Y-maze, Novel Object Recognition, and Rotarod performance comparisons of female WT mice (WT-F) and Plac1 X^{m}-X heterozygotes (HETs). Y-maze (n_{WT-F} = 7, n_{HET} = 4) revealed no significant differences in total number of entries (A), or percent of entries where the mice enters all three arms sequentially without revisiting a previously selected arm (percent alternation, B). In Novel Object Recognition (n_{WT-F} = 7, n_{HET} = 3), analysis revealed no significant change in percent time exploring the new object vs. the old (C) or ratio of time spent attending to the new object over the duration of the test (determination ratio, D). Rotarod (n_{WT-F} = 7, n_{HET} = 4) test showed no significant difference between genotypes with regard to time before falling off or performance improvement (E).
**Figure 13.** Radial Arm Water Maze (RAWM) test performance comparison of 16 mo. female WT mice (WT-F, n<sub>WT-F</sub> = 6) and 16 mo. Plac1 X<sup>m</sup>-X heterozygotes (HETs, n<sub>HET</sub> = 3). In the RAWM, mice are placed into a pool with six arms, with an escape platform at the end of one arm. After 15 trials, mice rest for one day. The subsequent day of testing begins with the exit in the same location as the final trial of the first day. WT-F and HETs perform similarly with regard to learning performance (A) and total errors (B). On the third day, the exit for each trial is opposite (180°) of the original location. There was no apparent difference between genotypes with regards to adapting to this reversal (C) or total errors during the reversal experiment (D). On the fourth day, the arms are removed from the pool, the platform is elevated, and a large flag is placed on the goal. Mice are tested in this fashion to ensure that the mice can see and are able to climb onto the platform. There were no deficiencies for any mice (E).
CHAPTER FOUR:

DISCUSSION

In this study, we find that Plac1 is expressed throughout the entire fetal brain during embryogenesis. Ablation of Plac1 results in histopathological abnormalities, including stunted hippocampal, cerebellar, and cortical growth, and reduced volume of the lateral ventricles. Furthermore, KOs show reduced neuronal and axonal expression, and an increase in microglial activation. Plac1 mutants have been previously shown to be at risk for lethal hydrocephalus (HC), with an 11% and 22% increase in risk for X<sup>m</sup>-X heterozygotes (HETs) and KOs respectively (Kong et al., 2013). L1CAM, a neural adhesion glycoprotein, serves as the genetic basis for most cases of X-linked HC (Jouet et al., 1993). L1CAM additionally has the capability to interact with FGF receptors, similar glycoproteins such as phosphaglycan, and its surrounding environment, to induce signaling cascades (Weller & Gartner, 2001). PLAC1 is a putative glycoprotein containing a zona pellucida 3 motif (Cocchia et al., 2000), a module important in protein-protein interactions and polymerization (Jovine et al., 2002). Although the mechanism of PLAC1 action is unknown, we speculate that it may interact with L1CAM-dependent developmental and regulatory pathways during embryogenesis.

Knowing that Plac1 ablation is associated with increased risk of fatal postnatal HC, we were surprised to observe that the knockout mice studied displayed restricted lateral ventricles compared to the WT control. X-linked HC is typically associated with enlargement of the lateral ventricles (Dahme et al., 1997; Fransen et al., 1998). There are a couple explanations for this
observation. First, there is considerable variability of observed phenotypes in genetic mouse models of disease that is dependent on factors such as mouse strain, genetic background, and environmental influences (Cohen et al., 1998; Dahme et al., 1997; Doetschman, 2009; Fransen et al., 1998; Kenwrick, Jouet, & Donnai, 1996). Second, Plac1 KOs show considerably reduced viability (Jackman et al., 2012). The studied KO may display a milder phenotype that allowed this individual animal to avoid developing HC. The exact mechanism of how Plac1 influences the development of X-linked HC remains to be elucidated.

We next attempted to determine if Plac1 ablation led to long-term phenotypic changes in adult mice. Although there were insufficient numbers of male KOs to study, we speculated that female HETs would likely be affected. The PhenoMaster system was used to begin characterizing the Plac1 phenotype. It is an automated high-throughput phenotyping platform that allows unobtrusive and low-stress observation and analysis of spontaneous behavior of several mice simultaneously. Although it has been recently proven to be a reliable and sensitive tool in behavioral analysis (Clemens et al., 2014; Urbach et al., 2014) extensive validation is still needed (Tecott & Nestler, 2004; van der Staay & Steckler, 2001). An increase in the number of screened mice over a greater range of ages could allow the use of multivariate models, such Partial Least Squares Discriminant Analysis, to better describe an overall Plac1-deficient phenotype and make greater use of the collected data.

Results from these studies revealed that HETs exhibited decreased activity and increased rearing during the acclimation phase at 5 months, and increased exploration, peripheral movement, and rearings throughout the entire experiment at 12 months, compared to female WT (WT-F) equivalents. These results suggest potential motor, anxiety, and memory-related deficiencies that we qualify by classical behavioral testing.
The elevated plus maze (EPM) and open field test (OF) are established tests used to assess state anxiety-related behavior in rodents (Hazim, Ramanathan, Parthasarathy, Muzaimi, & Mansor, 2014; Uys, Stein, Daniels, & Harvey, 2003). The OF test relies on the conflict between the exploration of novel environment and the fear of open spaces. The percentage of time spent in the center is the primary assay for anxious behavior (Prut & Belzung, 2003). Likewise, the EPM pits aversion to elevated open spaces against the innate tendency to explore. Percentage of time and number of entries into the open arms is used to assess anxiety and performance of anxiolytics (Lister, 1987; Uys et al., 2003). We show that while HETs perform identically to WT-F in the OF test, HETs have reduced head entries into open arms, do not explore the entirety of the open arms, exhibit reduced exploratory behavior, and spend more time immobile or frozen. Anxious behavior as seen on the EPM has been associated with knockout of serotonin transporter 5-HTT and serotonin receptor 1A (Holmes, Lit, Murphy, Gold, & Crawley, 2003; Ramboz et al., 1998), and decrease in GABA<sub>A</sub> neurotransmission (Andolina, Maran, Viscomi, & Puglisi-Allegra, 2014; Ishihara, Hiramatsu, Kameyama, & Nabeshima, 1993). This suggests *Plac1* may have a role in the development of serotinergic or GABAergic circuits.

The lack of genotype effect of *Plac1* on anxiety-related statistics in the OF test may be due to several reasons. First, the EPM is usually considered a more sensitive test of anxiety (Ramos, 2008). Second, it has been shown that EPM and OF measure different aspects of anxious behavior. The measures of stress response between these tests depended on distinct factors, and conflicting test results between OF and EPM is encountered often in pharmacological screening (Anchan, Clark, Pollard, & Vasudevan, 2014; Ramos, 2008; Ramos, Mellerin, Mormede, & Chaouloff, 1998; Vendruscolo, Takahashi, Bruske, & Ramos, 2003). Third, the OF test may not encompass all measures of anxiety (Prut & Belzung, 2003). Multiple
tests are often encouraged to gain a more comprehensive picture of rodent behavior (Ramos, 2008; van Gaalen & Steckler, 2000).

We show that HETs perform equivalently to WT-F on the Rotarod, suggesting coordination, balance, and motor learning are largely unaffected in HETs. Cerebellar performance, a key determinant of Rotarod results (Lalonde, Bensoula, & Filali, 1995), is probably unaffected in HETs. The decrease in locomotor parameters seen in PhenoMaster analysis and the EPM are likely related to exploratory or anxious behavior rather than an inability to move. Furthermore, satisfactory performance on the radial arm water open maze variant confirms that HETs have the ability to see and move properly. It would be interesting to test KOs on the Rotarod, as there was observable dysmorphia and neuronal depletion in the cerebellum of a Plac1 knockout that might hinder locomotion.

The Y-maze, novel object recognition (NOR), and radial arm water maze (RAWM) are all useful behavioral assays for uncovering deficits in memory, locomotion, and cognition. The Y-maze is typically employed as a measure of spatial working memory (Dridi et al., 2014). The NOR task is sensitive for cognition and recognition memory deficits (Grayson et al., 2014). The RAWM can be used to detect problems with spatial memory and learning (Alamed, Wilcock, Diamond, Gordon, & Morgan, 2006; Shukitt-Hale, McEwen, Szprengiel, & Joseph, 2004). WT-F and HETs performed similarly in all three tests, suggesting that HETs have no significant deficits in spatial memory, learning, or cognition. These performances are classically linked to hippocampal function (King, Trinkler, Hartley, Vargha-Khadem, & Burgess, 2004), implying HET hippocampi are largely normal. However, recent review proposes that certain aspects of anxiety are associated with the hippocampus (Bannerman et al., 2014). It is unclear how EPM-related anxiety results and spatial memory performance correlate.
Plac1 X<sup>m</sup>X heterozygotes appear to be spared from aforementioned gross anatomical malformations, adulthood neuronal loss, reduced axonal development and migration, and microglial activation. Although the paternal allele is largely silenced, the small amount of residual expression that escapes inactivation appears to provide some degree of functional compensation during development. This is consistent with the observed paternal imprinting of Plac1 in maternal-allele Plac1 mutants (Jackman et al., 2012). However, the increased susceptibility of HETs to HC, abnormal phenotypes of HET embryo and placenta (Jackman et al., 2012), and reported distinct behavioral differences, strongly suggests there may be some other manifestation of nervous system insult. Methods used for these studies may not have detected subtle structural differences between HETs and WT-F brains. The comparison can be expanded in several directions: incorporating more brain cell markers, such as astrocytes or oligodendrocytes, using Weil stain to check for myelin, including a wider array of mice brain ages, or analyzing certain neuronal circuits.

As the cellular function, location, and expression regulation of Plac1 expression have not been fully characterized, it is impossible to highlight a causative role for Plac1 in the development of the nervous system. It is possible the altered neural phenotype of Plac1 HETs and KOs may result from aberrations in placental nutrient transport rather than direct action of Plac1 in the developing brain. Recently, the placenta has been identified as an exclusive source of serotonin for forebrain development between E10.5 and E15.5 of mouse development (Bonnin et al., 2011). This is interesting given the association between Plac1 X<sup>m</sup>X heterozygotes and possible serotonin-related anxiety. However, the small but detectable presence of Plac1 in the embryonic brain suggests a more active role. Additionally, differential microarray analysis of E18.5 KO placentae revealed downregulation of several genes important in embryogenesis,
including Bmp, Wnt, and Pdgf (Fant, unpublished data). Heavily implicated affected canonical systems include “ES Cell Pluripotency”, “Integrin Signaling”, “Glioblastoma Multiform Signaling”, and “Axonal Guidance Signaling”, further suggesting a direct role for Plac1 in neural development. Selective tissue knockouts for Plac1 can help examine the relative importance of expression in these respective organs. Elucidation of PLAC1 function and potential protein interactions will shed light into Plac1’s role in neural development.
REFERENCES


APPENDIX A:

IACUC APPROVAL DOCUMENTATION

Procedures and protocols such as timed matings between mice were carried out in accordance with Institutional Animal Care and Use Committee (IACUC) of the University of South Florida College of Medicine Protocol Number R4228. The following pages are scans of IACUC approval documentation for the two years this study was conducted.
MEMORANDUM

TO: Michael Fant, M.D.
Dept. of Pediatrics
CR12008

FROM: Jay B. Dean, Ph.D., Chairperson
Institutional Animal Care & Use Committee
Division of Research Integrity and Compliance

DATE: 3/6/2013

PROJECT TITLE: The Role of Plac1 in Placental Development

AGENCY/SOURCE OF SUPPORT: March of Dimes
FY09-503

IACUC PROTOCOL#: R 4228

PROTOCOL STATUS: APPROVED

Your request for continuation of this study was received and will be reported to the Institutional Animal Care and Use Committee (IACUC) at its 4/2013 meeting. The IACUC acknowledges that this study is currently on-going as previously approved. Please be advised that continuation of this study is in effect for a one-year period beginning 4/27/2013.

In addition, please take note of the following:

- IACUC approval is granted for a one-year period at the end of which, an annual renewal form must be submitted for years two (2) and three (3) of the protocol. After three years all continuing studies must be completely re-described in a new application and submitted to IACUC for review.

- All changes to the IACUC-Approved Protocol must be pre-approved by the IACUC [IACUC policy III.11]. Minor changes can be submitted to the IACUC for review and approval as an amendment or procedural change, whereas major changes to the protocol require submission of a new IACUC application. Minor changes are changes considered to be within the scope of the original research hypothesis or involve the original species and are submitted to the IACUC as an Amendment or Procedural change. Any change in the IACUC-approved protocol that does not meet the latter definition is considered a major protocol change and requires the submission of a new application. More information on what constitutes a minor versus major protocol change and procedural steps necessary for IACUC review and approval are available on the Comparative Medicine web site at http://www3.research.usf.edu/cm/changes-protocol.asp

cc: Comparative Medicine
MEMORANDUM

TO: Michael Fant, M.D.
Dept. of Pediatrics
CRI2008

FROM: Jay B. Dean, Ph.D., Chairperson
Institutional Animal Care & Use Committee
Division of Research Integrity and Compliance

DATE: 3/1/2014

PROJECT TITLE: The Role of Plac1 in Placental Development

AGENCY/SOURCE OF SUPPORT: March of Dimes FY09-503

IACUC PROTOCOL#: R 4228

PROTOCOL STATUS: APPROVED

Your request for continuation of this study was received and will be reported to the Institutional Animal Care and Use Committee (IACUC) at its 4/2014 meeting. The IACUC acknowledges that this study is currently on going as previously approved. Please be advised that continuation of this study is in effect for a one-year period beginning 4/27/2014.

In addition, please take note of the following:

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cc: Comparative Medicine

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INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE
PHS No. A100-01, AAALAC No.58-15, USDA No. 58-15
University of South Florida · 12901 Bruce B. Downs Blvd., MDC35 · Tampa, FL 33612-4799
(813) 974-7106 · FAX (813) 974-7091