Analysis of the function of FNDC3B during mammalian development.

INTRODUCTION

The placenta provides nutrients and oxygen to the developing fetus and facilitates removal of fetal waste. Abnormal placental development and function can have serious consequences for the fetus that include spontaneous abortion, pre-eclampsia, premature birth, , and low birth-weight. One in thirteen babies born in the U.S. each year has low birth-weight (March of Dimes, 2005). Moreover, low birth weight infants can experience serious health problems as newborns, and are at an increased risk of long-term disabilities. Thus, understanding the mechanisms responsible for abnormal placental development is an important step towards the eventual goal of being able to diagnose and ameliorate such conditions.

Both environmental and genetic factors influence placental growth. Smoking, malnutrition and alcohol consumption during pregnancy can compromise placental function and alter placental morphology (Ashfaq et al.). In recent years, genetic analysis in mice has identified several novel genes that function in placental development (Cross et al.), including Mash2, insulin growth factor and peroxisome proliferator-activated receptor – gamma (PPAR-γ). PPAR-γ is a "master" regulator of adipocyte differentiation. PPAR-γ is expressed within the developing placenta where it is required for fatty-acid transport as well as trophoblast differentiation (Fig 1). However, it is currently unknown how PPAR-γ itself is regulated in the developing placenta.

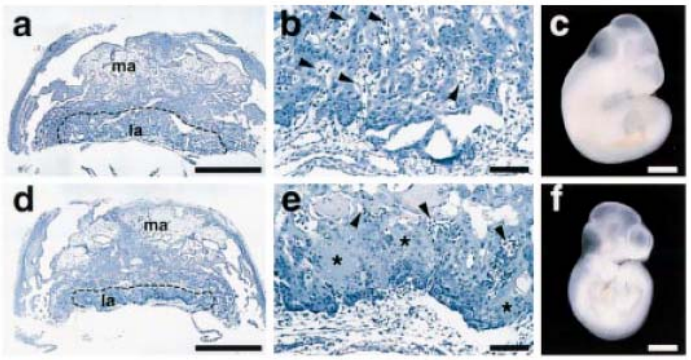

Fig 1. Placental defects in *PPARγ*-Deficient Mice Histopathology of placentae and embryos of *PPARγ*-deficient animals at 10.5 dpc. Sagittal sections of wild-type (a and b) and *PPARγ* (d and e) placentae are shown. Dotted lines in (a) and (d) show the boundary between the labyrinth layer and the spongiotrophoblast layer. Higher magnification of the labyrinth layer (b and e) reveals reduction in development of capillary vessels in placentae from *PPARγ* mutant conceptsuses, compared with wild type. The *PPARγ*–γ embryo (f), associated with the placenta shown in (d) and (e), showed overall growth retardation compared with wild-type embryo (c). Key - ma, maternal decidua; la, labyrinth layer. Arrowheads in (b) and (e) indicate fetal blood vessels (note that not all the vessels are marked). Bars in (a) and (d) indicate 1 mm. Bars in (b) and (e) indicate 100 mm. Bars in (c) and (f) indicate 5 mm. (From Kubota et al).

PRELIMINARY STUDIES

Studies in the MacGregor laboratory have shown that a gene known as *Fndc3*, found on mouse chromosome 14, plays a critical role in spermatogenesis and mammary gland development and function in mice (unpublished observations). *Fndc3* encodes a polypeptide that possesses three distinct structural domains: a proline-rich N-terminus, a central region containing nine fibronectin type III domains and a hydrophobic C-terminus. *Fndc3* is highly related to an additional gene in mice called Fad104. Recent studies suggest that FAD104 may be an upstream regulator of PPAR-γ (Fig 2).

Fad104 is expressed during differentiation of pre-adipocytes into adipocytes in culture. RNA—mediated reduction of Fad104 expression in pre-adipocytic 3T3-L1 cells blocks adipocyte differentiation (Tominaga et al. Fig2A). Significantly, reduced

expression of Fad104 results in decreased expression of PPAR-γ (Tominaga et al. Fig 2B).

Fad104 functions upstream of PPAR-γ in adipocyte differentiation in a cell line in vitro. **We hypothesize that *Fad104* is required *in vivo* for placental development where it is required to activate PPAR-γ.**

To test this hypothesis, the MacGregor laboratory has generated mice with a mutation within *Fad104*. The mutation is a gene-trap allele for *Fad104* gene ($Fad104^{XK507}$). The gene trap system allows one to mutate genes at random. It also facilitates analysis of where the mutated gene is expressed as the trapped (mutated) gene now makes β-galactosidase which can be identified using the chromogenic substrate X-gal (Fig 3).

To investigate the consequence of loss of function of FAD104 on mouse development, we dissected a pregnant heterozygous mutant $Fad104^{XK507/-}$ female at 14.5 days post-coitum (mouse gestation is approximately 20 days). The results revealed embryos with striking phenotypes (Fig 3). Of seventeen embryos, nine appeared healthy, three appeared to have died around embryonic day 10.5, and five extremely small conceptsuses were in advanced stages of reabsorption. We hypothesize that the nine normal appearing embryos are either heterozygous or wild-type for the gene trap mutation while the three embryos that died around embryonic day 10.5 are homozygous mutant for *Fad104*. Indeed, each of the embryos and placenta from the small embryos that appeared to have died at e10.5 stained blue, consistent with the possibility that they are homozygous mutant for the gene trap mutation in Fad104 (Fig 3). In contrast, 3 of 9 normal appearing embryos did not stain with X-gal and are wild-type for the mutation. These numbers are consistent with an expected mendelian ratio of 1 :2 :1 wild-type : heterozygous : homozygous mutant *Fad104* mice. To verify this result, PCR analysis of the nine normal appearing embryos was used to confirm that the three non-staining embryos did not contain the mutated allele (Fig 4). As expected, the remaining six possessed at least one gene-trap allele. The five extremely small conceptuses were in advanced stage of resorption and could not be analyzed by x-gal staining. We believe these embryos died simply due to over-crowding of the uterus.

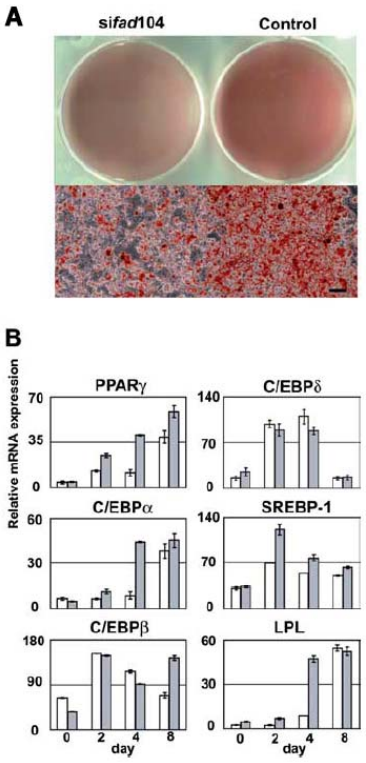

Fig. 2 Effect of reduced *Fad104* expression by RNAi on the differentiation of 3T3-L1 cells. (A) 3T3-L1 cells were transfected with a shRNA vector for *Fad104* in (si*fad104*, left panel) and a control shRNA vector (control, right panel). After eight days, the cells were fixed and stained with Oil red O. (B) Effect of reduced Fad104 expression on various adipogenic factors. Quantitative RT-PCR analysis was conducted to assay total RNA from sifad104 cells (White bars) and control (Gray bars). Q-PCR was used to normalize expression levels with 18S rRNA. (Tominaga *et al*)

We also examined the pattern of X-gal staining in the conceptsuses to determine if *Fad104* is expressed in the placenta during development. The results (Fig 3) suggest that *Fad104* is expressed in the placenta, possibly within the spongiotrophoblast and

labyrinthine layers. This result is particularly interesting as PPAR-γ is expressed in the labyrinthine layer of the placenta (Kubota et al).

In summary, the results of these preliminary studies are consistent with our hypothesis that *Fad104* functions within the placenta during embryogenesis, where it is required to activate PPAR-γ.

SUMMER RESEARCH PLAN

The goal of my research this summer is to continue my Bio199 studies into the function of *Fad104* in mouse development. I will attempt to accomplish three goals.

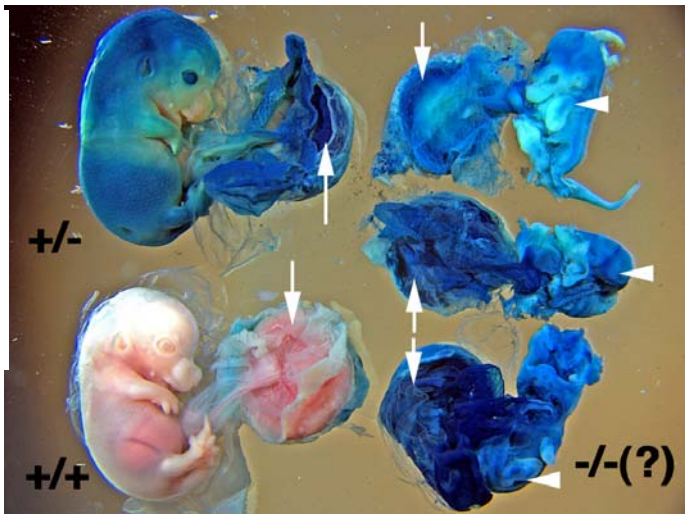

Figure 3. Embryos from a pregnant heterozygous mutant Fad104$^{XK507/-}$ female dissected at 14.5 days post-coitum. Embryos were fixed in 4% paraformaldehyde, and assayed for β-galactosidase activity with X-Gal. Representative embryos of wild-type (+/+), pGT0LXF hemizygote (+/-) and presumptive pGT0LXF homozyotes (-/-) are shown. Note the low background X-gal staining in +/+ embryo, and the relatively high B-geo activity in both the placentae (arrow) and embryo proper (arrowheads).

First, I will identify when and why *Fad104* homozygous mutant embryos die during development. To do so, I will generate heterozygous mutant female mice that have been mated with heterozygous mutant males. Pregnant females will be euthanized between 9 and 14 days pc and the conceptsuses dissected and analyzed by histology for evidence of embryonic lethality associated with abnormal placental development. My hypothesis predicts that the placental defects will be similar to those observed in PPAR-□ mutant mice. However, it is possible that the defects may be more subtle, or completely different.

My second goal is to identify the cell types in which *Fad104* is expressed during fetal development. To do so, I will analyze the pattern of expression of the trapped gene using X-gal staining of embryos and extra-embryonic membranes. To verify these results, I will also perform immunohistochemistry on histology using embryos and placentae from wild-type and mutant FAD104 animals using antibodies specific for mouse FAD104 that are currently

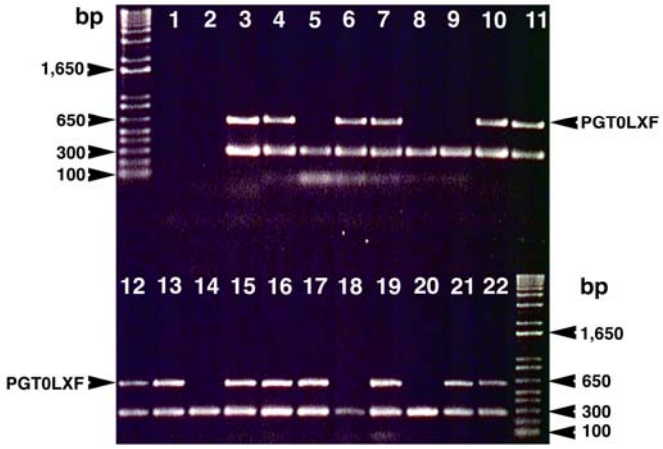

Figure 4. PCR-based assay to identify pGT0LXF (gene-trap) allele. Lane1: No Sample, Lanes 2 and 3: negative and positive controls, respectively. Lanes 4-10: Tail-extraction DNA samples from B6 neonates (not discussed in text). Lanes 11-22: Yolk-Sac extraction DNA samples from embryos removed from pregnant heterozygous mutant Fad104$^{XK507/-}$ female at 14.5 days post-coitum. Note – lower band (280bp) is positive internal control for PCR reaction. Upper band (608bp) is specific for gene trap allele.

being developed by the MacGregor laboratory.

Finally, I will test my hypothesis that PPAR-γ expression in vivo is dependent upon *Fad104*. To do so I will perform western analysis and immunohistochemistry on placentae from wild type and Fad104 homozygous mutant embryos. My hypothesis predicts that placenta from *Fad104* homozygous mutant embryos will lack, or have reduced, expression of PPAR-γ.

In conclusion, my proposed studies should provide new insight into the function of novel gene in mammalian development. These studies may be of use in understanding some of the reasons for low birth-weight, premature birth and miscarriage during pregnancy in humans.

RESPONSIBILITY

I will work under the supervision of Dr. Grant MacGregor, the lab PI, Ms. Katrina Waymire, the lab's Research Specialist, and Mr Kevin Obholz, the lab's senior graduate student. These individuals will train me to perform all of the techniques required for my project, including animal husbandry, establishing timed pregnancies, dissection of embryos, photography, embedding, preparing histology, examining and photographing pathology, extracting DNA, running PCR reactions for genotyping, extracting protein and running western and b-gal assays. I have already learned several of these methods (underlined) during my Bio 199 studies. Most importantly, I continue to improve my skills in how to build models, develop hypotheses and design experiments to test these hypotheses. I will interact with my professor on a daily basis and will give presentations of my work at least twice during the summer at the lab's weekly lab meeting. Given my progress to date, I am confident that I can accomplish my research goals within the summer period.

TIMELINE

Week(s)	Specific Aim	Experiments
1-8	Identify when FAD104-mutant mice die during embryonic development.	Generate pregnant heterozygous FAD104 females that have been mated with FAD104 +/- males, and isolate embryos. Analyze by histology.
2-9	Analyze morphology of placentae from FAD104 wild-type and -/- embryos to investigate whether there is abnormal development of specific cell types.	Perform histology and immunohistochemistry using probes to gene products known to be expressed in the developing placenta.
3-9	Analyze status of expression of PPAR-□ in placentae from FAD104 -/- and control embryos	Perform western and immunohistochemistry analyses of placenta from FAD104 +/+ and -/- embryos.
9-10	Write-up experiments and present findings to lab	

IACUC Protocol Number

The experiments described in this proposal are covered under UCI IACUC # 2004-2503 "Genetic Analysis of Mammalian Development", G. MacGregor PI.

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2. Cross, J.C. *et al* (2004) Genes and Development – A Workshop Report. *Placenta* **25**, Supplement A, Trophoblast Research, Vol 18, S39-S41.
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