LOSS OF THE MURINE TATA-BINDING PROTEIN N TERMINUS LEADS TO PLACENTAL LABYRINTH DEFECTS BUT NOT MATERNAL ADAPTIVE IMMUNE RESPONSES

by

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ABSTRACT

The TATA-binding protein (TBP) is a component of the basal transcription machinery. The TBP C-terminal core is conserved in all eukaryotes, but the N terminal region is shared between vertebrates. To study this, we generated a mouse line lacking 111 of the 135 amino acids of the vertebrate-specific sequence. The mutant *tbp* allele was designated $tbp^{\Delta N}$, and the majority of homozygous mutants, $tbp^{\Delta N/\Delta N}$, died at midgestation due to defects in the placenta. Previous studies in our laboratory showed that $tbp^{\Delta N/\Delta N}$ fetuses survived the midgestational crisis if the mother was severely immunocompromised or if mutant fetuses were supplied $tbp^{+/+}$ placentas. From these data, we hypothesized that the TBP N terminus regulates placental activity that is required for tolerizing the maternal immune system to the conceptus. Recent histological analysis of embryonic day 8.5 (E8.5) to E12.5 placentas in immune wildtype mothers revealed that the $tbp^{\Delta N/\Delta N}$ placental vascular region, or labyrinth, does not develop. In the severely immunocompromised, *rag1*-deficient mothers, the labyrinth forms, but the vasculature appears to be reduced. Morphometric analysis of E14.5 mutant placentas from *rag1*-deficient mothers, showed a 2-fold reduction in the number of labyrinth blood vessels. These results suggest that the TBP N Terminus is involved in labyrinth formation and vascularization. Since *rag1*-deficient mice lack functional B and T cells, we wanted to determine which adaptive immune cells might be involved in the rejection. The $tbp^{\Delta N}$ mouse line was bred to mice lacking various adaptive immune cell subsets to determine which cells were necessary for $tbp^{\Delta N/\Delta N}$-specific rejection. However, no adaptive immune cell subsets were found to be required for the rejection process. Further maternal immune system characterization via FACS analysis of maternal para-aortic lymph nodes and careful histological analysis showed that maternal adaptive immune cell populations did not change with response to $tbp^{\Delta N/\Delta N}$ fetuses and no adaptive immune cells appeared to infiltrate into the placenta prior to or during rejection. Therefore, the $tbp^{\Delta N}$-specific labyrinth malformation is most likely triggering an innate immune pathway.

CHAPTER 1

INTRODUCTION

The TATA-Binding Protein

The Role of TBP in Basal Transcription

Transcription is the process by which organisms transfer genetic information from chromosomal DNA to messenger RNA (mRNA). This process is highly regulated to guarantee the correct timing and manner of genome expression. Initiation of transcription is controlled by the presence of multiple macromolecular complexes at a given promoter (Zhao and Herr, 2002). In eukaryotic transcription, RNA polymerases (RNAPs) cannot directly recognize target promoters. The RNAPs I, II, and III, instead, require specific accessory factors, including the TATA-binding protein (TBP) and general transcription factors TFIIA, B, D, E, F, and H, to interact with the promoter region in order for polymerases to initiate transcription. In the case of TBP, the protein helps recruit RNAPs either directly by binding to the DNA consensus sequence TATAa/tAa/t within the promoter region known as the TATA box, or indirectly through protein-protein interactions with proteins that bind promoters lacking a TATA box (Berk, 2000; Hernandez, 1993; Roeder, 1996).

The importance of TBP in transcriptional initiation is best understood through *in vitro* and *in vivo* studies of RNAP II in TATA-containing promoters. Transcriptional initiation begins when TBP binds to the TATA box forming a

highly stable DNA/protein complex that unwinds the DNA region and brings upstream and downstream TATA elements closer to the promoter region. This process is expedited if TBP binds TFIIA as a member of the TFIID complex. Once the TBP/DNA complex is formed, TFIIB is able to bind to TBP as well as the DNA regions upstream and downstream of the TATA box. This, in turn, results in the recruitment of the preformed complex RNAP II/TFIIF that binds to TFIIB. This large multimeric initiation complex is completed with the addition of TFIIE, H, and J and RNA template synthesis proceeds upon the addition of ribonucleotide triphospates (Buratowski and Zhou, 1988; Hernandez, 1993; Roeder, 1996, Sawadogo and Sentenac, 1990)(Fig. 1). Without the initial binding of TBP to the TATA box in these systems, the transcription machinery is not recruited and mRNA is not synthesized, showing that DNA-binding by TBP is an integral rate-limiting step in the process.

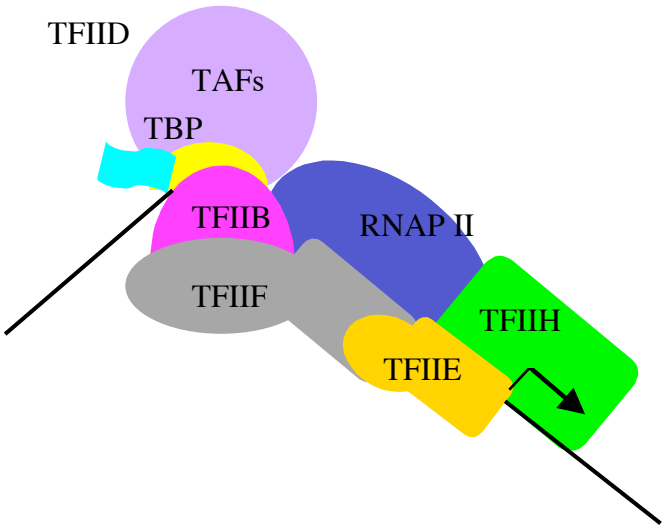
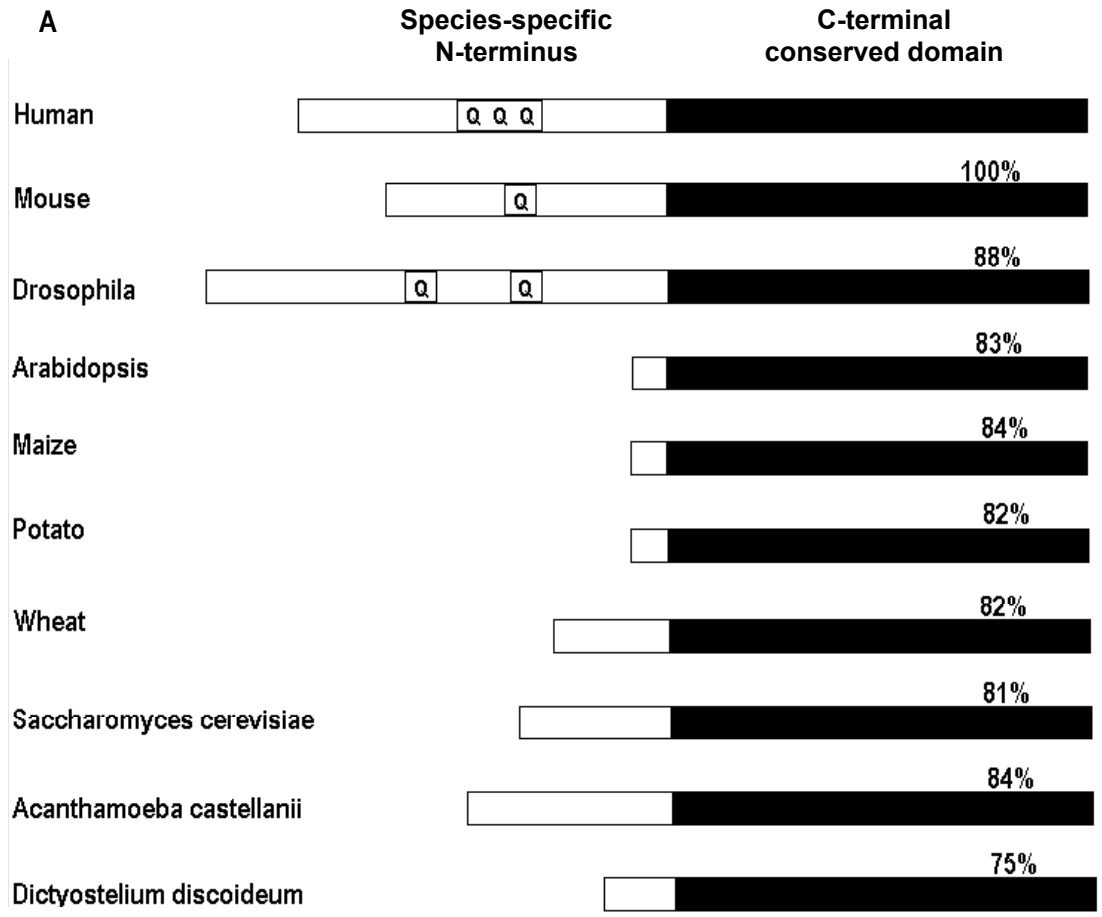


Figure 1. The preinitiation complex.

Structure, Function, and Evolution of TBP

TBP proteins from most metazoan animals have two domains; a conserved carboxy- (C-) terminal core domain and a species-specific amino- (N-) terminal domain (Um, et al., 2001). The C-terminal core consists of 180 amino acids and is evolutionarily conserved from Archaebacteria to man (Hernandez, 1993; Rowlands, et al., 1994; Bondareva and Schmidt, 2003) (Fig. 2 A). Data regarding this domain comprises most of what is understood about TBP. Studies in mouse and chicken cell lines indicate that the eukaryotic C-terminus is sufficient for TBP TATA-binding and DNA-bending activity as well as for all interactions in known complexes such as TFIID (Hernandez, 1993; Horikoshi, et al. 1990; Schmidt, et al., 2002; Um, et al., 2001). Crystal structures of the C-terminus show that the region regulates transcription by how it binds DNA and with which proteins or protein complexes it interacts (Hernandez, 1993; Littlefield, et al. 1999; Thomm, 1996). Null mutations of the C-terminus have been shown to block transcription in yeast and mice resulting in yeast inviability and embryonic lethality at embryonic day 3.5 (E3.5) following depletion of maternal mRNA stores (Horikoshi, et al., 1990; Martianov, et al., 2002; Poon, et al., 1991). Limited knowledge of the N-terminus is primarily due to the lack of sequence conservation between phyla as well as the lack of an N-terminal crystal structure from which the function might be inferred (Hernandez, 1993) (Fig. 2 A and B). Previous studies, however, suggest that the eukaryotic N-terminus plays a role in regulating transcription. These studies include: biochemical experiments



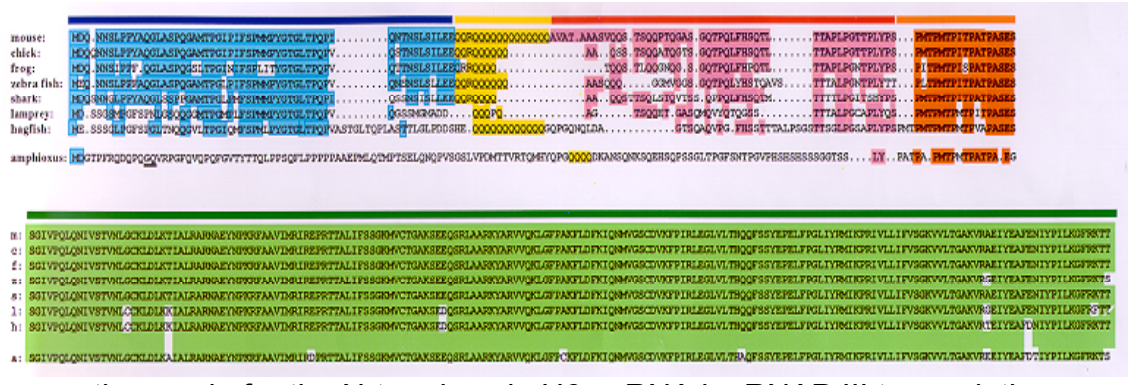


Figure 2. Species speficity of the TBP C and N terminus. **A.** Representation of TBP that shows the N-terminus (white) and C-terminus (black) along with percent amino acid conservation of the C-terminus. Adapted from Hernandez, 1993. **B.** Amino acid alignment of TBP N-terminus from higher vertebrates and to complex non-vertebrates. Top line, different regions of the N-terminus (blue, yellow, pink, and orange); bottom line, the C-terminus (green). Lucas, 2005.

suggesting that the N-terminus regulates activities of the TBP C-terminus by modulating C-terminal DNA-bending and TATA-binding activity (Henry, et al., 1995), and *in vivo* experiments in vertebrate cells suggesting the TBP N-terminus is necessary for full expression of genes involved in preventing cell death and regulating cell growth rates (Schmidt, et al., 2002; Um, et al., 2001). Homologous amino acid sequence between vertebrates (Fig. 2 B) suggests that the N-terminus has a vertebrate-specific function, which leads to the question: what role does the TBP N-terminus fulfill in vertebrates (Bondareva and Schmidt, 2003; Hernandez, 1993)?

The *tbp* Gene Family

In the yeast *Saccharamyces cerevisiae,* it was discovered that TBP was encoded by a single gene, and no other TBP-like proteins could be found in the organism (Hansen, et al., 1997). This finding led to the assumption that other organisms would also have only one TATA box-binding protein. However, recent studies have shown that TBP-related factors (TRFs) exist in a number of higher eukaryotes. The first TRF identified was designated TRF1 and has only been found in *Drosophila melanogaster.* Like TBP, TRF1 can bind the TATA box as well as TATA-less promoter sequences (Hansen, et al., 1997; Davidson, 2003). The protein also appears to have evolved as a replacement for TBP in RNAP III transcription and can act as an alternative initiator for some RNAP II transcribed genes. With genes that have more than one promoter, such as the *Drosophila* Tudor gene, TRF1 adds another layer of transcriptional regulation by binding to

the upstream promoter, while TBP selectively binds to the downstream promoter (Davidson, 2003; Holmes and Tjian, 2000).

A second TRF, found in all metazoans, has a variety of names-TRF2 (Rabenstein, et al., 1999; Teichmann, et al. 1999), TBP-related protein (TRP) (Moore, et al., 1999), TBP-like factor (TLF) (Dantonel, et al., 1999), and TBP-like protein (TLP) (Ohbayashi, et al., 1999). TRF2 (as designated here) has ~40% identity with the C-terminal core domain and lacks any substantial N-terminal sequence. However, the role of TRF2 in transcription is not well understood because TRF2, even with its similarity to the TBP C-terminus, does not bind the TATA box, and the DNA sequence that the protein does bind remains unidentified (Dantonel, et al., 1999; Davidson, 2003). *In vivo* experiments using *Caenorhabditis elegans, Xenopus laevis* (frog)*, Danio rerio* (zebrafish)*,* and *Mus musculus* (mouse) have tried by RNA interference (RNAi), antisense oligonucleotide, dominant negative, and targeted mutagenesis strategies to shed light on the function of TRF2. In *C. elegans*, frogs, and zebrafish, the loss of TRF2 leads to an early arrest in embryogenesis (Dantonel, et al., 2000; Kaltenbach, et al., 2000; Muller, et al., 2001; Veenstra, et al. 2000). This embryonic lethality is caused by blocked zygotic transcription of specific genes that require TRF2 for expression. Loss of TRF2 in mice, however, does not lead to embryonic lethality; rather, *trf2*$^{-/-}$ mice complete embryogenesis and develop into healthy adult animals (Zhang, et al., 2001). These mice, though, do have a tissue-specific phenotype associated with spermiogenesis that leads to male

sterility (Davidson, 2003; Martianov, et al., 2001; Zhang, et al. 2001). These functional studies suggest that TRF2, like TRF1, evolved to govern gene- and tissue-specific expression patterns in a TBP-independent fashion. Yet, unlike TRF1, TRF2 appears also to have evolved vertebrate-specific specializations.

Finally, TRF3 (or TBP2) is the most recently identified TBP-related factor (Bartfai, et al., 2004; Persengiev, et al., 2003). This *tbp* family member is found in vertebrates ranging from pufferfish to humans (Bartfai, et al., 2004). In humans, TRF3 has a C-terminus that is 93% identical to the TBP and binds the TATA box and TFIIA and B. This suggests that *trf3* evolved from a duplication of *tbp* (Persengiev, et al., 2003). TRF3 is also similar to TBP in that it has a fairly divergent N-terminus, sharing only 15% identity with the TBP N-terminus (Persengiev, et al., 2003). Sequence analysis of various vertebrate species shows that this domain is primarily comprised of species-specific sequence flanked by small vertebrate-specific domains. Currently, little has been published about this newest *tbp* family member. What is known is that TRF3 can initiate RNAP II transcription of certain genes and that in zebrafish, *trf3* and *tbp* are differentially expressed during embryogenesis and gonad development (Persengiev, et al., 2003). This may provide a means for tightly regulating activation of pertinent developmental genes. Also, human studies show that TRF3 enters the nucleus after TBP following mitosis suggesting that TRF3 may also participate in a temporal mechanism to reinitiate post-mitotic transcription (Bartfai, R. et al., 2004; Persengiev, et al., 2003).

The Placenta

Evolution of the Placenta

Embryonic development in modern day reptiles, birds, and mammals (amniote vertebrates) is a result of a new evolutionary direction that occurred over 300 million years ago (Blackburn, 1998; Gilbert, 2000). This evolutionary transition gave some reptiles the selective advantage of reproducing young in protective eggs. These oviparous species were, therefore, not limited to aquatic-associated terrains and were free to explore and live in new environments that other species could not. This freedom led to further niche-associated specializations that eventually resulted in viviparous embryonic development within some species. To support viviparity, extraembryonic tissue that originally supported nutrient, gas, and waste exchange between the embryo and egg evolved new adaptions for transporting the same materials between mother and embryo resulting in first primitive placentae (Gilbert, 2000). This transition from oviparity to viviparity occurred on numerous occasions in mammals. With each transition, a new type of placenta was formed, creating tremendous diversity in placental structure and function between mammalian orders (Stewart and Thompson, 2000) (Fig. 3). Phylogenetic analyses of these various types of

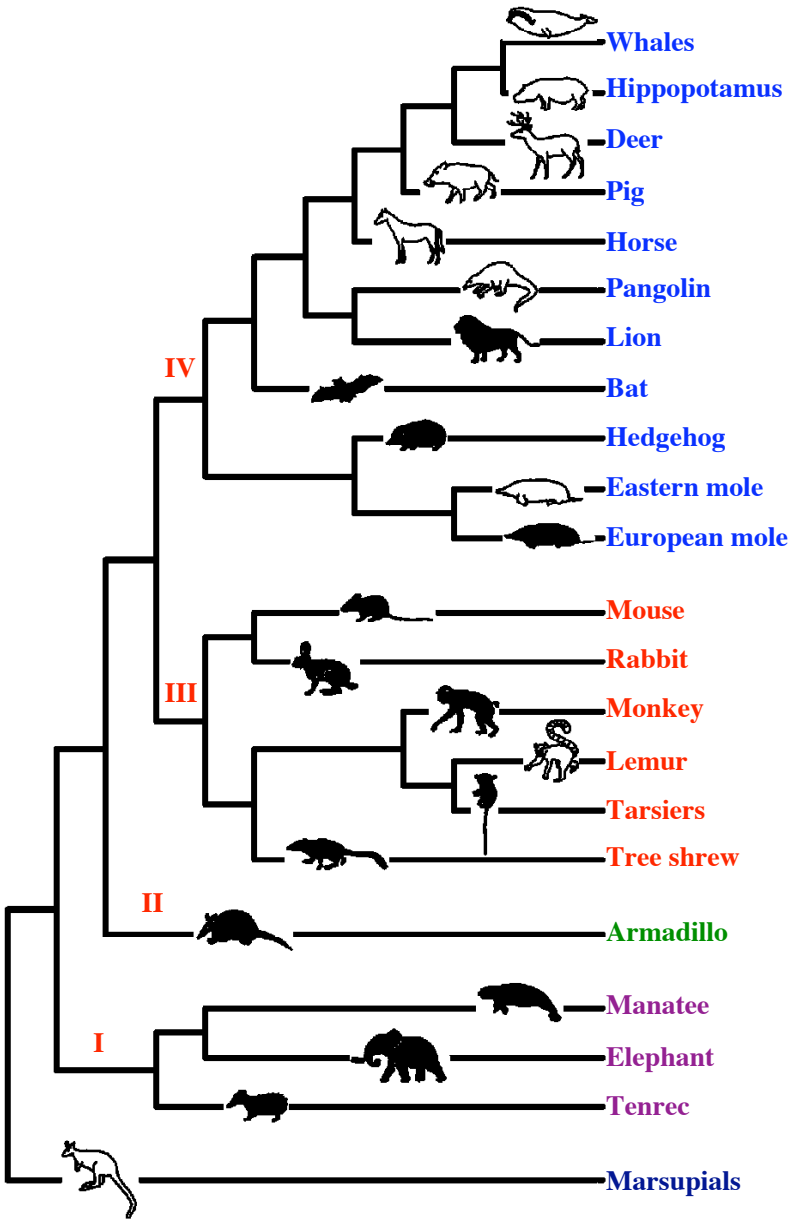


Figure 3. Phylogenetic tree of eutherian placental development. The species depicted here show the diversity in placental structure and function found in eutherian mammals. I-IV represent the four major eutherian clades. Adapted from Vogel (2005).
.

placentas show that they differ in the degree of maternal tissue loss, the histology of the fetal/maternal interface, gross morphology, and nutritional requirements (Vogel, 2005). However, in this tremendous diversity, some fundamental properties remain universal between these vertebrate taxa. All eutherian placentae have two main functions: (1) to interact with the uterus and secrete growth factors, hormones, and cytokines, and (2) to create a large surface area for nutrient exchange. Secreted factors affect the maternal physiology by promoting an increase in blood flow and nutrients to the fetal/placental tissue. These nutrients are transported to the chorioallantoic placenta, a region comprised of an epithelial barrier and an underlying system of fetal blood vessels. The epithelial layer prevents direct contact of the maternal and fetal blood only allowing nutrients to cross the barrier. Once across the barrier, the nutrients are transferred to the fetal blood vessels and on to the fetus (Adamson, et al., 2002; Cross, et al., 2003a; Georgiades, et al., 2002).

The ancestral form of the chorioallantoic placenta in egg-laying vertebrates is composed of two vascularized extraembryonic membranes: the yolk sac and the chorioallantoic membrane. The yolk sac receives nutrients from the yolk, and in modern egg-laying vertebrates such as birds and reptiles, it is required throughout embryonic development. In eutherian mammals, the yolk sac is somewhat vestigial, but it still supplies nutrients to the early embryo prior to placental formation (Cross, et al., 2003a; Cross et al., 1994). The chorioallantoic membrane is generated by the fusion of the chorion and allantois

membranes (Cross, et al., 2003b). Though this process is a characteristic of amniotes, the role of the resulting membrane varies drastically between species that utilize amniotic eggs and those with viviparous gestation (Blackburn, 1998; Cross, et al., 2003a). In avian species, the chorioallantoic membrane is structurally simple and thin. It adheres to the eggshell where the membrane participates in gaseous exchange and transfers calcium from the shell to the fetus. The chorioallantioc membranes in higher mammals, such as mice and humans are much more complicated and have unique functions that promote intrauterine embryogenesis. These include the ability to modulate the uterine environment using hormones and, in some species, the devlopment of trophblast cells which invade the uterine wall to establish more direct contact with uterine cells (Cross, et al., 2003a).

The Function, Structure, and Development of the Mouse Placenta

The mouse placenta provides a transport surface for oxygen and nutrients, it controls the maternal endocrine system to promote maternal blood vessel growth to the implantation site, and it fosters maternal immune tolerance during pregnancy (Cross, et al., 2002a; Hemberger and Cross, 2001). To establish this intimate system, cells from the embryo invade the uterus, leading to the eventual formation of four distinct placental layers. Maternal-derived placental cells form the first tissue layer called the maternal decidua. This tissue has direct contact with the uterine wall and is composed of specialized cells whose progenitors surrounded the original implantation site. Other layers, known

as the giant trophoblast layer, the spongiotrophoblast layer, and the labryinth layer, are generated from embryonic-derived cells, or trophoblasts (Fig. 4). The trophoblast cells are derived from extraembryonic tissue, and trophoblast giant cell precursors invade the uterine wall during implantation (Cross, et al., 2003a) (Fig. 5) The following paragraphs describe the formation of these placental layers.

The Mouse Blastostocyst and Implantation. Within 24 hours of fertilization (E1), the mouse zygote has undergone its first cell division (Fig. 6) Following this first division, these first two cells, or "blastomeres," continue to divide roughly every 12 hours. At the eight cell stage (E2), a phenomenon known as compaction occurs where the loose arrangement of undifferentiated cells huddle together, maximizing their contact with one another. This forms a condensed sphere where the outer cells form tight junctions isolating the inner ones from the external environment, thus, dividing the cells into two groups (Fig. 6). By E2.5, the developing embryo has 16 cells and is now referred to as a morula (Fig. 6). During the morula stage, the delineated groups of cells become two functionally and morphologically distinct cell types: the inner cell mass (ICM) blastomeres and the outer cell blastomeres, collectively called the trophoblast. By the 64-cell stage (E3.5), the morula has developed into a blastocyst with a fluid filled central cavity pushing the ICM to one side of the developing embryo (Fig. 6). At this point, the first differentiation event occurs in mammalian development as the ICM

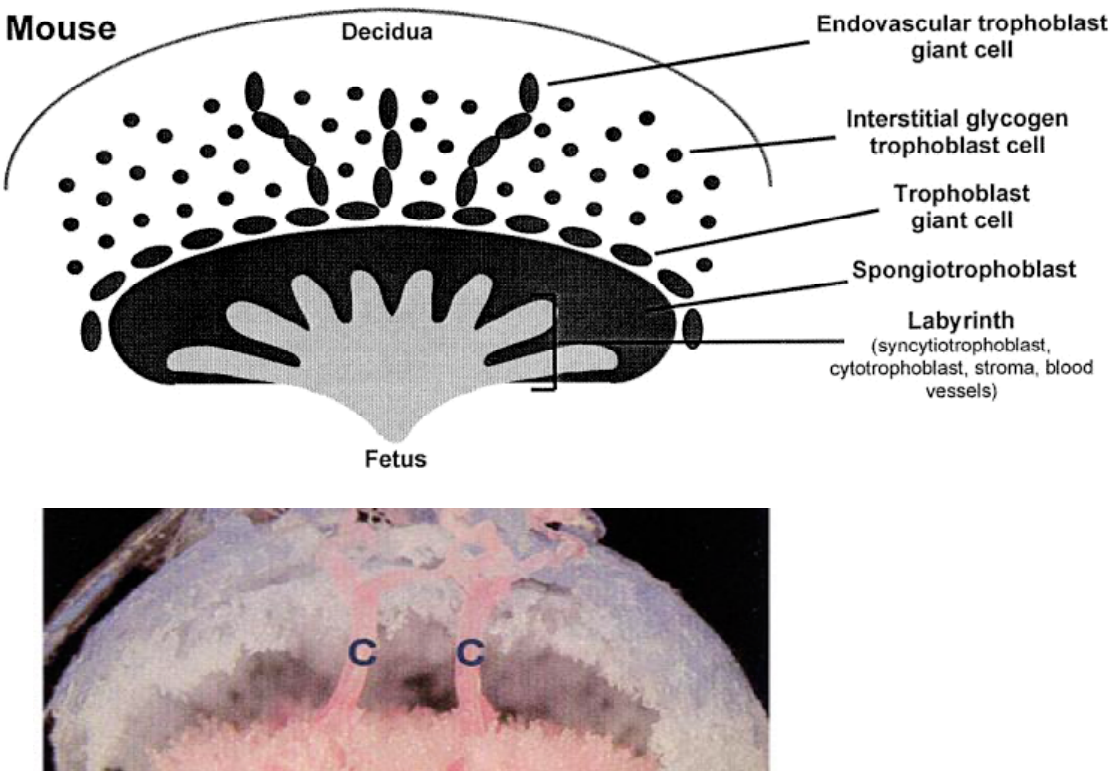


Figure 4. The developing mouse placenta. Top, anatomy of the mature mouse placenta. Bottom, mid-sagittal section through a placenta casted in blue plastic via the maternal vena cava and in red plastic via the maternal aorta; C, arterial canals. Adapted from Cross, et al., 2003a and 2003b.

and trophoblast cells form two separate layers whose cells cannot contribute to each other. The ICM cells are fated to give rise to the embryo proper and its associated yolk sac, allantois, and amnion, while the trophoblast-derived layer, or "trophectoderm," will develop into extraembryonic tissue creating the zygote-derived portion of the placenta (or chorion) (reviewed in Carlson, 1996 and Gilbert, 2000). The embryo, though, does not have the autonomous ability to

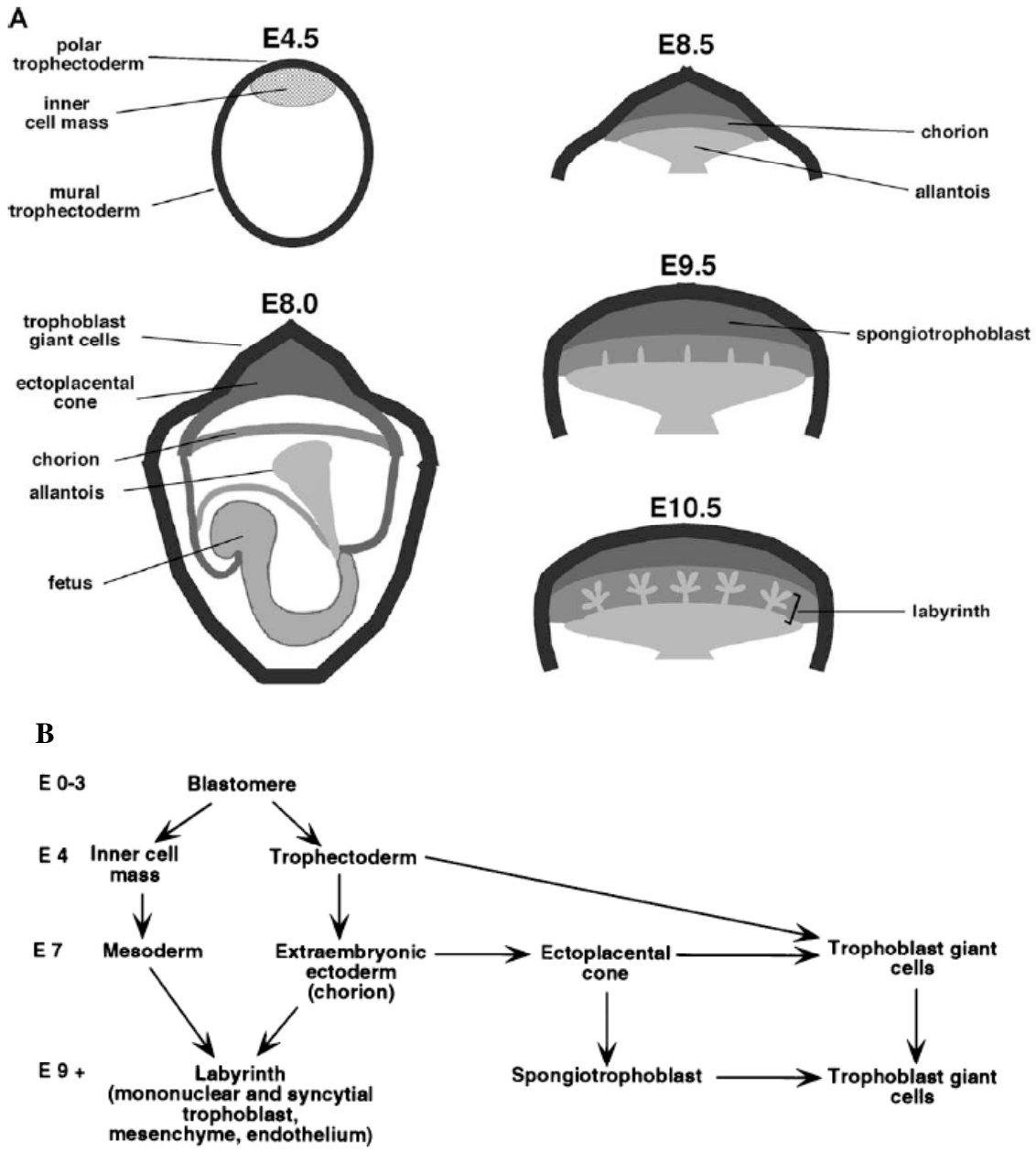


Figure 5. The developing mouse placenta. A. Placental development in mice from implantation to labyrinth maturation. B. Cellular differentiation for placenta-associated cells from the blastomere stage to mid-gestation and beyond. Adapted from Cross, 2005.

support the new developing tissues and growth. Therefore, it implants into the uterus so that the mother can provide the essential nutrients.

Before the mouse blastocyst can be implanted, the zona pellucida must be removed. To this point, this egg-derived membrane has remained intact to protect the developing embryo from the maternal environment, as well as to prevent premature adherence of embryonic cells to the oviduct as they travel to the uterus (reviewed in Carlson, 1996). By E4, a hole in the zona is opened from which the blastocyst squeezes out, causing the embryonic cells to be exposed to the uterine environment.

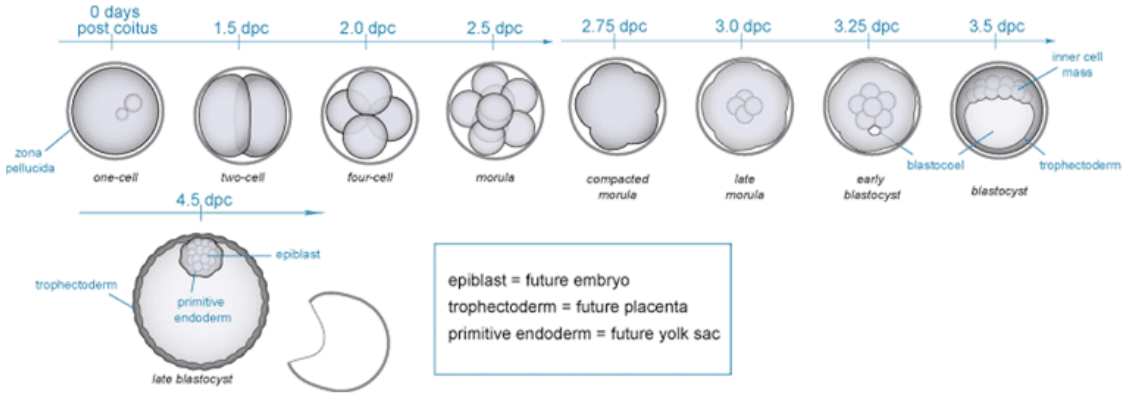


Figure 6. Mouse embryonic development.

The Maternal Decidua. The maternal decidua is a major component of the mouse placenta and develops as the result of implantation. Implantation occurs when the blastocyst alters a region of the uterine wall by contacting, attaching to, and invading its endometrium. (reviewed in Dey, et al., 2004 and Lee and DeMayo, 2004). The trophoectoderm closest to the ICM adheres to uterine

epithelial lining, primarily via integrin interactions with collagen, fibronectin, and laminin found on the extracellular matrix of the epithelial cells (reviewed in Gilbert, 2000). The tethered epithelial cells are then phagocytosed by blastocyst cells, and an opening is created through which the blastocyst cells can invade the uterus (reviewed in Lee and DeMayo, 2004).

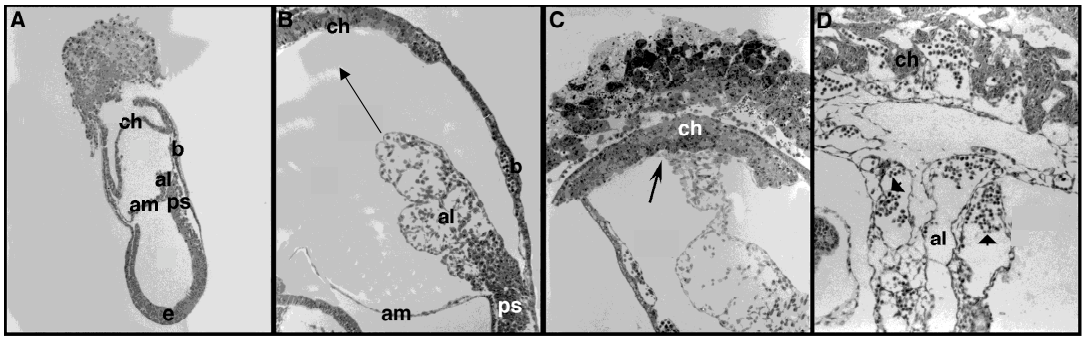
By E5.5, blastocyst cells move into the endometrial stroma. Cell-to-cell contact between the embryonic and stromal cells during this period eventually results in increased stromal cell proliferation, causing the murine uterus to increase in size by five-fold. Endometrial cells in direct contact with the blastocyst differentiate into cells that make up the primary decidual zone (pdz). Those adjacent to the pdz but away from the blastocyst form the secondary decidual zone (sdz) (Hunt, et al., 1997; Tan, et al., 1999). Around E9, both the pdz and the sdz undergo regression and reorganization, allowing the developing placenta to be re-positioned with the maternal decidua juxtaposing the mesometrium.

The mesometrium is a highly vascularized tissue, and blood vessels between it and the placenta allow embryonic tissue access to nutrition from the maternal blood supply. To promote vessel formation, uterine natural killer (uNK) cells home to the impantation site (Gu, et al., 1994; Hunt, et al., 1997). These cells produce cytokines that dilate existing maternal vessels and aid the growth of new ones. By E10.5, the uNK cells travel to the mesometrial side of the decidua forming a lymphocyte-dense area called the metrial gland. The metrial

gland regulates the final vascularization steps in the maternal decidua (further discussion of the uNK cells can be found in later sections). The mature blood vessels, known as spiral arteries, begin in the mesometrium, travel through the decidua, and end at arterial canals in the chorion placenta (Adamson, et al. 2002). The completion of these vessels marks the end of the decidualization process.

Trophoblast Giant Cell Layer. Trophoblast giant cells (TGCs) are the first specialized trophoblast cells to develop in the chorion placenta. These cells are unique because they are the only embryo-derived cells to maintain direct contact with the maternal blood supply and endometrial tissue through most of gestation. This maternal contact gives TGCs the ability to mediate implantation and invasion of the blastocyst into the uterine wall as well as to control local and systemic maternal physiology through hormone and cytokine production. TGCs regulate maternal blood flow, lactogenesis, ovarian progesterone production, and the maternal immune system (Cross, 2005).

Giant cells also have a distinctive morphology that make them easily recognizable. Mature TGCs are three to five times larger than other placental cells and have giant nuclei. These giant nuclei are due to endoreplication during differentiation. In this process, the TGC progenitors exit the mitotic cell cycle, increase in size, and begin DNA replication without cytokinesis, leaving the cells extensively polyploid (Cross, 2005; Goncalves, et al., 2003; MacAuley, et al., 1998; Zybina and Zybina, 1996).



Formation of the allantoic bud. Neural plate stage (7.25 - 7.5 dpc).

ps-primitive streak
b-yolk sac blood islands
am-amnion

Growth of the allantois. Early somite stage (8.0 - 8.25 dpc).

Chorioallantoic fusion (8.5 dpc). The arrow points to the chorioallantoic fusion junction.

Vascularization of the allantois. 9.5 dpc conceptus showing overt vascularization of the umbilical component of the chorioallantoic placenta. Arrowheads indicate embryonic red blood cells.

Figure 7. Chorioallantoic fusion. Electromicrographs of chorioallantoic fusion from E7.5 to E9.5 (ps-primitive streak, b-yolk sac blood islands, and am-amnion). Adapted from Downs, et al. 1998).

During early implantation, TGCs start to differentiate from blastocyst cells as part of the first steps toward chorion placenta development. As previously stated, the implanted blastocyst is initially composed of two single-cell layers, the ICM and the trophectoderm (Fig. 7a). All specialized trophoblast cell types found in the mature chorion placenta originate from this simple trophectoderm. By implantation (E4.5), the polarization of the ICM and contact with the uterine wall splits the trophectoderm into two regions, one which apposes the implantation site, the mural trophectoderm, and the other which lies above the ICM, the polar trophectoderm. TGCs arise from both of these layers but at different times during gestation (Cross, et al., 1994; Cross, 2000a; Cross, 2005; El-Hashash and Kimber, 2004; Goncalves, et al., 2003).

Because of differences in development, TGCs are divided into primary and secondary cell types. At E5.5, primary TGCs arise from the mural trophectoderm where they differentiate directly from the 50 or so mural cells. In early embryogenesis, these cells mediate implantation and invasion into the uterine wall. During placental development, primary TGCs are part of blood sinuses in the parietal yolk sac. This tissue is the first structure in the placenta to form, and while the placenta is forming, its sinuses facilitate O_2 and nutrient diffusion between maternal blood and the embryo. In the mature placenta, the primary TGCs produce part of the hormones and cytokines that regulate the maternal environment (Cross, et al., 1994; Goncalves, et al., 2003; Zybina and Zybina, 1996).

The secondary TGCs arise indirectly from the polar trophectoderm. As a result of implantation (E4.5), the polar trophectoderm proliferates and differentiates into trophoblast stem cells. Upon fibroblast growth factor 4 (FGF4) stimulation, this stem cell population expands and clusters to form a thick, conical-shaped region adjacent to the decidua, which is called the ectoplacental cone (EPC)(Rossant and Cross, 2001). First seen at E7, secondary TGCs are thought to originate from stem cells in the core of the EPC. By E9, spongiotrophoblast cells are believed to develop from this same stem cell population, replacing the EPC as a source for secondary TGCs.
Secondary TGCs are generally thought to have one main function-to secrete cytokines and hormones for regulating the maternal environment. However,

recent studies suggest that specialized giant cells called endovascular TGCs are a subset of secondary cells. Endovascular cells associate with the spiral arteries where they connect to arterial canals, marking the transition from endothial-lined vessels to trophoblast-lined blood spaces. If endovascular cells are a type of secondary TGC, then not only would secondary cells secrete factors to promote vascularization and increase blood flow to the conceptus, they would (like primary cells) have a physical connection with the placental vascular network (Adamson, et al., 2002; Cross, et al., 1994; Cross, 2000a; Cross, 2005; El-Hashash and Kimber, 2004; Goncalves, et al., 2003).

TGC differentation is regulated by both intrinsic and extrinsic signalling pathways. Intrinsically, the cell differentiation is controlled by a series of basic helix-loop-helix (bHLH) transcription factors. Members of this family interact via HLH domains, allowing the two basic regions of the proteins to bind DNA. Family members include *Mash2*, *Hand1*, and *Stra13*. *Mash2* suppresses TGC differentiation with *Mash2* mutant mice having concepti that arrest at E10.5 due to placental defects (Guillemot, et al., 1994). The mutant placentas have a boosted number of differentiated TGCs, a premature loss ectoplacental cone and spongiotrophoblast cells, and a poorly developed labyrinth layer (discussed below). *Hand1* and *Stra13*, on the other hand, promote TGC formation. *Hand1* mutants arrest at E7.5, blocking TGC differentiation and forming a smaller ectoplacental cone (Riley, et al., 1998). HAND1 likely competes with MASH2 in binding placenta-specific DNA targets, possibly off-setting each other in placental

development. *Stra13* is a retinoic acid-inducible transcription factor that is induced during TGC differentiation (Hughes, et al., 2004). Treatment of trophoblast stem cells with retinoic acid or over-expressing *Stra13* stimulates TGC formation (Cross, 2005; Hughes, et al., 2004; Scott, et al., 2000).

A key extrinsic factor that regulates TGC differentiation is tissue oxygen level (Adelman, et al., 2000). Oxygen is a potent regulator in placental vascular development (Adelman, et al., 1999; Phillips, et al., 1995), and hypoxia-inducible factor-1 (HIF-1) is a member of the bHLH family control pathway associated with oxygen deprivation (Wenger and Gassmann, 1997). HIF-1 is composed of two proteins, HIF-1alpha and ARNT. By binding to hypoxia response elements (HREs) in the promoter or enhancer regions of genes, HIF-1 heterodimer mediates the transcriptional response to the loss of oxygen and activates glycolytic, glucose transport, and angiogenic pathways (Wenger and Gassmann, 1997). Loss of these HIF-1 pathways, as occurs in *Arnt*-deficient mice, results in a non-viable placenta with an increased number of TGCs and a diminished spongiotrophoblast layer (Adelman, et al., 2000; Cross, 2005).

Spongiotrophoblast Layer. As previously mentioned, TGC development occurs in parallel with spongiotrophoblast cell development. The mature spongiotrophoblast layer (or junctional zone) forms the middle portion of the placenta, wedged between the TGC region and the innermost labyrinth layer. The function of the spongiotrophoblast is not well characterized; however, it is thought to play a structural role and is known to secrete factors promoting its own

maintenance and function. For example, spongiotrophoblast cells produce anti-angiogenic factors that suppress TGC-expressed vascular endothelial growth factor (VEGF) to prevent the spongiotrophoblast layer from being penetrated by the maternal endothelium (Cross, et al., 2002b; Cross, 2005).

Spongiotrophoblast cells most likely arise from stem cells within the ectoplacental cone. This belief is largely supported by gene expression data where genes, such as *Tpbp* and *Flt1*, that are expressed in the apex of the ectoplacental cone between E7.5 and 8.5 are later seen in the spongiotrophoblast layer but not in TGCs or the labyrinth layer (Cross, et al., 2003a; Cross, 2005). Recent findings published by Slevin, et al., 2006 further substantiate this genealogy by showing that fluorescent beads injected into the ectoplacental cone at E7.5 are found in spongiotrophoblast cells at E11.5.

As the spongiotrophoblast layer matures, secondary TGCs (as discussed in an earlier section) and glycogen trophoblast cells appear to differentiate from a subset of cells within the tissue. First seen around E12.5, glycogen trophoblast cells can be distinguished from spongiotrophoblast cells because their accumulated glycogen stains periodic acid Schiff (PAS) positive. Like TGCs, glycogen positive cells invade and embed into the maternal decidua, allowing them to establish cell-to-cell contact with maternal cells. These cells, though, do not make up or interact with decidual blood vessels as giant cells do, rather they migrate past the giant cells into the distal region of the decidua basalis where they reside for the remainder of the pregnancy as the main souce of insulin

growth factor 2 (IGF2) in the placenta (Adamson, et al., 2002; Cross, et al., 2003a; Cross, 2005; Redline, et al., 1993; Simmons and Cross, 2005). Currently, the role of these specialized trophoblast cells is unclear. However, it is known that the glycogen trophoblast cells continue to express spongiotrophoblast-specific markers throughout gestation, and IGF2 as well as cyclin-dependent kinase inhibitor C1 (p57 or cdckn1c) mutant mice show differences in differentiation, proliferation, and glycogen production in the cell types (Lopez, et al., 1996; Takahashi, et al., 2000).

Labyrinth Layer. The last placental structure to develop is the labyrinthine or labyrinth layer. This layer is an intricate, trophoblast-derived vascular network, and by E10, the labyrinth replaces the parietal yolk sac as the site for nutrient and gaseous exchange between mother and fetus. When first established, the labyrinth layer comprises a relatively small portion of the placenta. However, as the fetus grows, the layer expands and becomes more elaborate in response to the fetus's growing nutritional needs. By parturition, this region is the dominant placental feature, making up approximately two thirds of the placental mass (Cross, 2005; Simmons and Cross, 2005).

The labyrinth arises from the fusion of the mesoderm-derived allantois and the trophoblast-derived chorion. These two cell layers are spatially separated by the amnion in early development. At E7.5, the chorion represents a plate of cells arising from and adjacent to the EPC, and the allantois begins to rise toward the chorion as a small bud of cells originating from embryonic

primitive streak. (Fig. 7a). By E8, the allatois has doubled in length and ascends toward the chorion (Fig. 7b). Once in proximity (E8.5), the two layers fuse, thus triggering the differentiation of the chorionic trophoblast cells (Fig. 7c). In the next few hours (from E8.75 to E9), the chorion and the allantois interdigitate and rearrange to form the villi of the labyrinth vasculature. These rearrangements continue until the majority of the labyrinth as well as the umbilical blood vessels are formed (E9.5)(Fig. 7d). From this point, the allantoic- and chorionic-derived populations expand and branch to create the massive vessel bed required to support the growing fetus (Cross, 2005; Downs, et al., 1998; Simmons and Cross, 2005; Stecca, et al., 2002).

A number of genes are known to regulate labyrinth genesis. These include the mouse homolog for *Drosophila* glial cell missing *(gcm1*)(Stecca, et al., 2002), *brachyury (t*)(Rashbass, et al., 1991*),* the LIM homeobox protein1 gene *(lhx1* or *lim1*)(Shawlot and Behringer, 1995), the vascular cell adhesion molecule 1 gene (*vcam1*)(Gurtner, et al., 1995; Kwee, et al., 1995*),* α*4-integrin* (*itga4*)(Yang, et al., 1995*), DnaJ (Hsp40) homolog, subfamily B, family 6 (dnajb6*)(Hunter, et al., 1999), the fibroblast growth factor receptor 2 gene *(fgfr2*)(Xu, et al., 1998*),* and the bone morphogenetic proteins 5 and 7 gene (*bmp5/7*)(Solloway and Robertson, 1999). Loss of any of these genes leads to either a malformed or non-existent labyrinth layer.

gcm1 is currently the most characterized of these genes with respect to labyrinth development. *gcm1*-null mice reveal that Gcm1 controls labyrinth

formation and vascular branching (Anson-Cartwright, et al., 2000). mRNA and protein analyses show that *gcm1* expression delineates three stages of chorio-allantoic interaction (Anson-Cartwright, et al., 2000; Schreiber, et al. 2000). Gcm1 mRNA, but not protein, is first detected in the pre-fusion chorion between E7.5 and E8. Upon chorion/allantoic fusion, a strong, cell-specific mRNA and protein signal can be detected at the chorionic/allantoic interface (E8.5). By E10.5, both signals are substantially fainter and found dispersed throughout the labyrinth in chorion-derived cells. Therefore, it is not surprising that *gcm1* is commonly be used as a marker in assays such as *in situ hybridization* to distinguish labyrinth from other placental layers and to gage progress in labyrinth development (Stecca, et al., 2002).

The Role of the Immune System during Pregnancy

The Maternal Immune System

Successful pregnancies are not immunosuppressive states in which maternal and fetal cells are sequestered into cell-impermeable barriers. Rather, maternal and fetal cells move across the placenta's maternal/fetal interface throughout gestation while the maternal blood supply has direct contact with trophoblast cells in the labyrinth vascular network (Luppi, 2003). Such contact results in immune cells and various immune factors of the innate and adaptive immune systems which recognize fetal antigens (Morgan and Holmes, 2000; Tafuri, et al.,1995). In a healthy pregnancy, the maternal and trophoblast cells

have pregnancy-supportive mechanisms that help tolerize the maternal immune system to the conceptus, thus neutralizing attacks on the fetus (Petroff, 2005). However, when the maternal immune system is unchecked, the fetus is often rejected (Cross, et al., 2002a; Hemberger and Cross, 2001; Moffett-King, 2002; Pandey, et al., 2005).

Innate Immunity. Many miscarriages share features with an innate immune response. Like innate responses, they have rapid activation, little or no specificity, and no memory as well as inflammation, complement activation, and leukocyte infiltration, usually precede or accompany abortion-related events. Thus, it is not surprising that recent evidence from animal models suggests that many abortions may be triggered when an innate immune response is activated or an innate regulator is disrupted (Caucheteux, et al., 2003; Moffett-King, 2002).

During mammalian pregnancy, the major maternal immune cell found within the decidua is the uNK cell (Kanellopoulos-Langevin, et al., 2003). uNK cells are innate immune cells with various critical roles in the placenta that include protecting the conceptus from invading pathogens and supporting placental development. During a microbial infection in humans, uNK cells that recognize Toll-like receptor (TLR) agonists respond to the infection by secreting interferon-γ (IFN-γ) and by upregulating TLR expression and related pathways (Eriksson, et al., 2006a; Eriksson, et al., 2006b). Most of the time, though, the uNK cells are involved in regulating decidualization, producing pregnancy-sustaining cytokines, and interacting with trophoblast cells to moderate both cells'

activity (Murphy, et al., 2005). In mouse models lacking uNK cells, the supportive nature of the cells are clearly visible as fetuses die around E12.5 due to placental defects in spiral artery formation and trophoblast invasion. Injections of IFN-γ into NK cell-deficient, pregnant mice through midgestation can block this lethal phenotype, thus showing that uNK cell IFN-γ is vital for placental development (Ashkar and Croy, 2001).

uNK cells do not always protect and nurture the placenta. As seen in IL-10 knockout mice, these cells can become hyperstimulated by TLR agonist such as LPS to promote rather than hinder destructive inflammatory responses in the placenta. The result is uNK-associated fetal resorption or intrauterine growth restriction triggered by even low doses of a TLR agonist (Murphy, et al., 2005).

Complement is the other arm of the innate immune system that plays a crucial role in pregnancy success. The complement system is utilized to directly lyse cells while activating and recruiting inflammatory cells (Caucheteux, et al., 2003). Normally, this process is reserved for pathogens or damaged tissue; however, if proteins that regulate complement are not present during pregnancy, complement activation can lead to widespread necrosis of healthy tissue (Caucheteux, et al., 2003; Kohl, 2006).

Necrosis is possible because the placenta is exposed to low levels of complement through contact with the maternal blood supply. Experimental evidence using semi-allogeneic pregnancies suggests that, prior to or during abortions, necrotic lesions (likely caused by stress or ischemia) lead to localized

inflammation or hemorrhaging (Caucheteux, et al., 2003; Mellor, et al., 2001; Munn, et al., 1998; Xu, et al., 2000). This tissue destruction exposes circulating complement to fetal alloantigens (or antibodies attached to alloantigens), thus activating the complement cascade and initiating leukocyte recruitment.

Under normal circumstances, trophoblast cells restrict the low level complement activity by expressing complement regulators, such as complement receptor1-related gene/protein y (Crry) and indoleamine 2,-3-dioxygenase (IDO)(Caucheteux, et al., 2003; Mellor, et al., 2001; Munn, et al., 1998; Xu, et al., 2000). These regulators restrict complement either by binding directly to complement proteins or by blocking upstream signals that activate the complement cascade, and the loss of a regulator results in a similar pattern of necrosis-associated fetal death.

Crry directly blocks complement by preventing actived C3 and C4 from depositing on autologous cell surfaces (Caucheteux, et al., 2003; Cross, 2000b; Xu, et al., 2000). Crry is expressed in maternal vascular-associated trophoblast cells and maternal decidua. Mouse embryos lacking *Crry* (*crry*$^{-/-}$) die between E10.5 and E14.5 due to activated complement lysing the endothelial-like trophoblast cells that normally express Crry. This embryonic lethal phenotype, however, can be rescued by generating and intercrossing compound mutant mice that are *crry*$^{+/-}$*; c3*$^{-/-}$ (homozygous null complement C3 mutation) or by injecting the mother with soluble recombinant Crry (Cross, 2000b; Holers, et al., 2002; Xu, et al., 2000).

IDO, on the other hand, indirectly hinders the complement cascade by degrading an essential amino acid, tryptophan (Trp)(Munn, et al., 1999). Trp is a key regulator of cell proliferation, and IDO, which is expressed by antigen-presenting cells (APCs), macrophages, and trophoblasts during gestation, is believed to breakdown the amino acid in order to suppress T cells that recognize alloantigens (Munn, et al., 1998). IDO activity can be chemically blocked *in vivo* by injecting a pregnant mouse with 1-methyl-tryptophan, and loss of IDO activity correlates with a loss of fetuses between E7.5 and E9.5 due to vascular trophoblast cell lysis. IDO inhibition, however, does not appear to induce abortion in dams that are homozygous mutant for recombination activation gene 1 (*rag1*$^{-/-}$)(Mellor, et al., 2001). Further characterization of this mouse model has found that cytolytic CD8^{+} T cells, but not B cells, control the complement cascade, suggesting that an alternate complement pathway is neutralized by IDO activity (Caucheteux, et al., 2003; Mellor, et al., 2001).

Adaptive Immunity. The placenta contains a variety of adaptive immune cells speckled throughout its different tissue layers. These cells include CD4^{+} $\alpha\beta$, CD8$^{+}\alpha\beta$, CD8^{+} $\gamma\delta$, and NKT cells, and a few of these cells are usually visible in the decidua of a healthy placenta at any time during gestation (Arck, et al. 1997; Robertson, 2000).

Previous studies using the stress-abortion-prone mouse model DBA/2-mated CBA/J show that the relationship between specific subsets of these decidual T cells can determine the outcome of the pregnancy (Arck, et al. 1997;

Robertson, 2000). For example, abortion-prone females not immunized against abortion events accumulate TNF-α and IFN-γ producing decidual $\gamma\delta$ T cells beginning at the peri-implantation period. Antibody depletion of the $\gamma\delta$ T cells prevents the abortive process (Arck, et al. 1997). However, females immunized against abortion with BALB/c male splenic cells (immunologically foreign) have a boosted population of decidual $\alpha\beta$ T cells, and antibody depletion of the $\alpha\beta$ T cells leads to abortion (Arck, et al. 1997). Therefore, when the female is not immunized against abortion, T_H1 decidual $\gamma\delta$ T cells predispose the pregnancy to abortion; whereas, when the female is immunized, decidual $\alpha\beta$ T cells are necessary for immunological tolerance to the pregnancy (Arck, et al. 1997; Robertson, 2000). This reveals that adaptive immune cell-mediated processes can regulate abortion and that a balance between antagonistic adaptive immune responses may be required to maintain a successful pregnancy.

The maternal adaptive immune system, however, does not simply control abortive processes by modulating its response to alloantigens during pregnancy. Syngeneic studies also have pointed to the role of adaptive immune cells like Vα14 NKT cells in abortion. Vα14 NKT cells accummulate in the decidua of syngeneic pregnancies (Ito, et al. 2000). This NKT cell subset uses CD1d, which does not require β_2M for cell surface express and is activated via alpha-galactoceramide (αGalCer) (Burdin, et al., 1998). αGalCer is a glycolipid antigen that stimulates the Vα14 NKT cells in a possible response to parasites or an altered maternal environment during pregnancy. Once Vα14 NKT cells in the

dam are activated, the embryos are aborted during implantation as trophoblast cells invade the maternal decidua (Ito, et al., 2000).

Statement of the Hypotheses

The evolutionary transition from the invertebrate to the vertebrate body plan required a large number of new genes and an enormous increase in transcriptional regulation. In the midst of this arising genetic diversity, selective evolutionary pressures prompted even the highly conserved basal transcriptional machinery to be altered to accommodate new communication pathways. In the case of the basal transcription factor, TBP, the protein gained a new N-terminal domain that has since remained intact in all vertebrates. We hypothesize that this vertebrate-specific TBP N terminus represents a transcriptional regulator that coevolved with vertebrate-specific genes to modulate their activity.

By generating and studying a line of mice that lack most of the TBP N terminus (*tbp*$^{\Delta N}$), our laboratory has determined that homozygous mutant (*tbp*$^{\Delta N/\Delta N}$) fetuses die at midgestation due to a placental defect(s). These fetuses survive through midgestation in mothers with severely compromised adaptive immune systems or when they are simultaneously homozygous mutant for β_2*m,* suggesting that the maternal immune system recognizes the placental defect in a β_2M-dependent manner. This β_2M-dependent pathway, though, is most likely not associated with classical MHC I response since no memory responses is induced by *tbp*$^{\Delta N/\Delta N}$ fetal rejection. From the mouse-specific data, we hypothesized that

the TBP N terminus regulates placental activity that is required for tolerizing the maternal immune system to the conceptus.

First, I present data on our analyses of the morphological changes that occurred in the placenta prior to and at the point of *tbp*$^{\Delta N/\Delta N}$ rejection. Histological analysis revealed defects in the labyrinth region (i. e. reduction of vascularization), which may be important for understanding the underlying causes of *tbp*$^{\Delta N/\Delta N}$-specific midgestational lethality. Then, I describe how changes in the maternal immune system affect *tbp*$^{\Delta N/\Delta N}$ survival at midgestation. Results suggest that, no matter what arm of the adaptive immune system is removed, a compensatory pathway or an innate immune response may still be present to reject *tbp*$^{\Delta N/\Delta N}$ fetuses. I next compared the immune systems of mothers that were carrying or not carrying *tbp*$^{\Delta N/\Delta N}$ fetuses and found that no dramatic shift in the maternal immune system occurred as a result of *tbp*$^{\Delta N/\Delta N}$ fetal death or labyrinth defects. These new findings, along with previous data, have led us to two conclusions: (1) the vertebrate-specific TBP N terminus regulates labyrinth development in mice (and possibly all eutherian mammals) and (2) the maternal adaptive immune system is not directly involved in *tbp*$^{\Delta N/\Delta N}$-specific immune rejection, but may be regulating innate immune responses pathways against *tbp*$^{\Delta N/\Delta N}$ placentas.

CHAPTER 2

METHODS

The *tbp*$^{\Delta N}$ Mouse Line

The *tbp*$^{\Delta N}$ mouse line was generated using targeted homologous recombination where a targeting vector was designed to replace genomic sequence encoding 111 of the 135 vertebrate-specific amino acids in the TBP N-terminus with two copies of the FLAG epitope tag (Fig. 8)(Bondareva and Schmidt, 2003; Hobbs, et al., 2002). The design conserved all splicing signals, introns, and promoters (Ohbayashi, et al., 1996; Schmidt, et al., 1997). Targeting was performed in mouse strain 129/J embryonic stem (ES) cells, and these were transferred into blastocysts of C57Bl/6J mice to generate chimeras. Southern blot and polymerase chain reaction (PCR) analyses were used to isolate targeted clones from random insertions by testing neomycin resistant cells for the presence of the MC1-*neo* cassette and FLAG tag, the absence of *tbp* exon 3, and the correct genomic context. ES cells that carried the appropriate *tbp* mutant allele were injected into C57Bl/6 mouse blastocysts creating chimeric mice. Those chimeric mice that carried a germline copies of the *tbp*$^{\Delta N}$ mutation were mated to generate *tbp* heterozygous (*tbp*$^{\Delta N/+}$) animals (Hobbs, et al., 2002).

To prevent the possible influence of the *neo* selectable marker on the phenotype of the *tbp* mutants (Fiering, et al., 1995), the Cre/*loxP* recombination system was used to remove the *neo* cassette. The targeting vector was

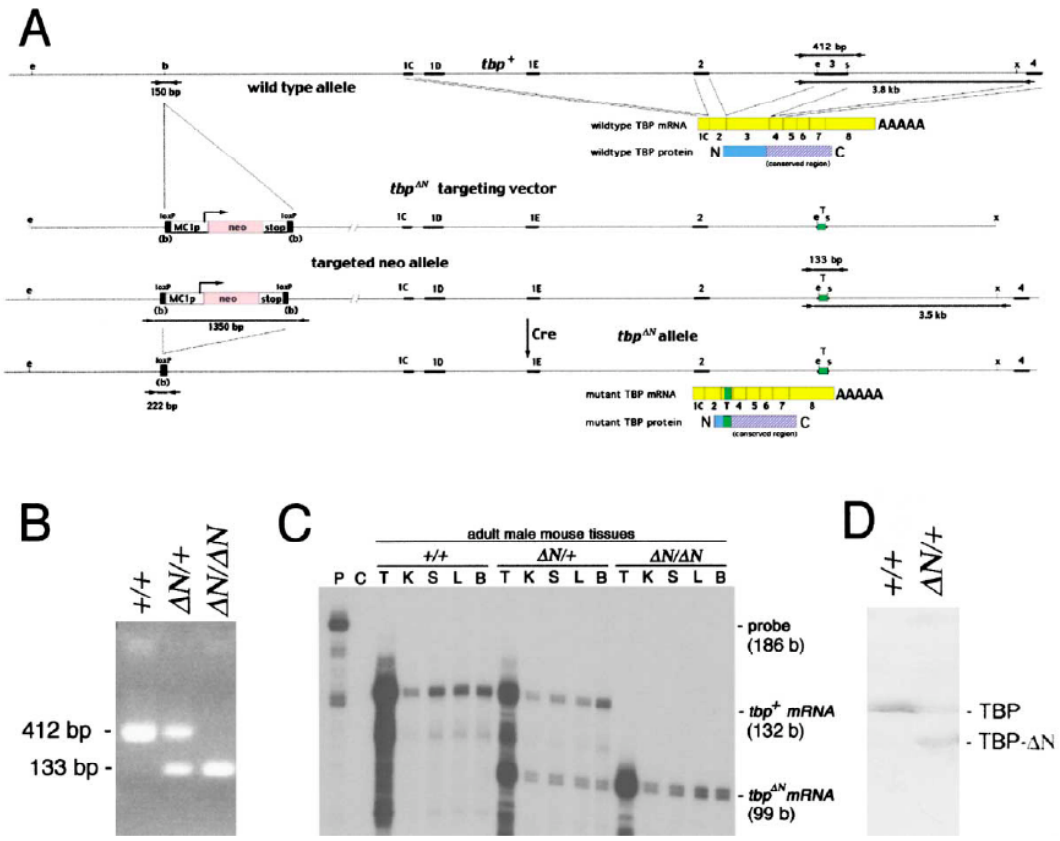


Figure 8. Targeted mutagenesis of N-terminal protein-coding sequences of the mouse *tbp* gene. **A.** Targeting strategy and vector design. The 5'end of the wild-type mouse tbp gene ($tbp^{\Delta N}$) is diagramed (fine horizontal line on top) indicating 5' exons (thickened regions of line, labeled 1C, 1D, and 1E for alternate promoter/first exons, 2, 3, and 4), selected restriction sites (b, BamHI; e, EcoRI; s, SacI; x, XhoI), diagnostic PCR primers (arrows), and PCR product sizes (region between arrows, lengths indicated). Below is indicated the predominant splicing pattern (gray broken lines) that yields the predominant TBP mRNA (yellow) and cognate TBP protein product (blue box). The N and C termini are indicated. Below is shown the targeting vector design, including the replacement of most of exon 3 with two tandem copies of the FLAG epitope tag (green box). Below this is indicated the targeted tbp allele still containing the *lox*P-flanked MC1p-neo gene, with the sizes of diagnostic PCR fragments from the primers shown above on the *tbp* allele indicated. At the bottom is shown the targeted allele after removal of the *lox*P-flanked MC1p-neo gene by Cre recombinase, with the resultant expressed somatic cell TBP mRNA and TBP protein indicated below. **B.** Genotyping animals using the primer set that spans the N mutation. **C.** Expression of TBP and TBP- N mRNA in mouse cells and tissues from adult male mice (8- to 12-weeks-old). Positions of undigested probe, TBP mRNA, and TBP-ΔN mRNA are indicated at right. Abbreviations: P,1:100 dilution of undigested probe; C, control lane containing probe hybridized to 50 μg yeast RNA; T, testis; K, kidney; S, spleen; L, liver; and B, brain. **D**. Wildtype and mutant TBP protein expression in adult mouse spleen nuclei (right). Figure published in Hobbs, et al., 2002.

designed so that MC1-*neo* was flanked by *loxP* sites (Gu, et al., 1993; Hobbs, et al., 2002). The final *tbp* mutant construct possessed only a *loxP* remaining from the recombination event and produces a mutant protein that has two FLAG epitope tags, replacing amino acids 25-135 of the TBP protein (Fig. 8).

Genotypic Analysis

All genotypes were determined using genomic DNA collected from tail snips obtained at weaning or fetal tissue. Tails were digested overnight at 55°C in 400 μL of 50mM Tris-Hcl (pH 7.5), 100mM EDTA, and 1% SDS with 100 μg/mL Proteinase K (PK). NaCl was added to each sample bringing the final concentration of salt to 2M. The digested tails were then placed on ice for 30 minutes or incubated at 4°C for 1 hour followed by a 5 minute centrifugation in a microfuge at 13,000 rpm at 4°C . The supernatant of each tail was poured into a tube with 1 mL of 100% Ethanol (EtOH) precipitating the genomic DNA. The DNA pellet was recovered and transferred to 200 μL of 1mM Tris-Hcl (pH 7.5) and 500mM EDTA. For each fetus, two samples, usually a hand and a foot, were digested by adding the tissue to 200 μL of 50mM Tris-HCl (pH 7.5), 100mM EDTA, and 1% SDS with 20 μg/mL PK. Samples were then digested overnight at 55°C, and NaCl was added to each sample resulting in a final concentration of 500mM. To precipitate the genomic DNA, 500 μL of 100% EtOH was added directly to the tube. The digested tail mixtures were vortexed, and the resulting DNA pellets were transferred to 100 μL of 1mM Tris-Hcl (pH 7.5) and 500mM

EDTA. Genotypes were determined for each fetus and weaned mouse by performing PCR. The alleles assayed included: *tbp*, *rag1,* β_2*m, cd8, ifgr, tcr*β*,* and *tcr*δ. All PCR constructs were amplified on an Eppendorf Mastercycler, and PCR products were separated on a 1.5% agarose gel in 1XTBE with ethidium bromide so that DNA bands representing mutant and wildtype alleles could be visualized when exposed to UV light.

tbp Genotypes

Two primer sets were used to amplify *tbp* genomic DNA. The first primer set amplifies the *tbp* exon 3 region. Exon 3 encodes the majority of the TBP N-terminal sequence and is replaced by two FLAG epitope tags in the $tbp^{\Delta N}$ mutant construct. Amplification of the wildtype and mutant alleles resulted, respectively, in a 412 base-pair and a 133 base-pair product. The second primer set amplified a region of the *tbp* gene that is upstream of the exon 1 promoters. In mutant alleles, this region contained either a *loxP*-flanked MC1-*neo* cassette or one *loxP* (after Cre recombination). The wildtype allele resulted in 150 base-pair product, the MC1-*neo* mutant allele produced a 1,350 base-pair product, and the *loxP* only mutant allele resulted in a 222 base-pair product. The PCR program used for both primer sets was 94°C for 30 seconds; 32 cycles of 94°C for 20 seconds, 61.5°C for 30 seconds, and 72°C for 2 minutes; and one cycle at 72°C for 5 minutes.

rag1 Genotypes

The *rag1* mutation is detected using three primer sets. The wildtype-specific primer set amplifies a 1,549 base-pair product and the mutant-specific primer set amplies a 1,300 base-pair product. The primers were amplified using the PCR protocol 94°C for 30 seconds, 35 cycles at 94°C for 30 seconds, 63°C for 30 seconds, and 72°C for 2 minutes 20 seconds; and one cycle of 72°C for 5 minutes.

β_2*m* Genotypes

The β_2*m* primer sets was a three primer set that gave a wildtype product of 374 base-pairs and a mutant product of 1,524 base-pairs. The products were amplified using the following PCR protocol: one cycle at 94°C for 30 seconds, 32 cycles at 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 2 minutes 30 seconds; and one cycle of 72°C for 5 minutes.

cd8 Genotypes

The *cd8* (*lyt-2*) primer set gave a wildtype product of 160 base-pairs and a mutant product of 1,300 base-pairs. The products were amplified using the following PCR protocol: one cycle at 94°C for 30 seconds, 32 cycles at 94°C for 20 seconds, 58°C for 30 seconds, and 72°C for 2 minutes; and one cycle of 72°C for 5 minutes.

ifgr Genotypes

The *ifgr* primer set gave a wildtype product of 197 base-pairs and a mutant product of 1,500 base-pairs. The products were amplified using the following PCR protocol: one cycle at 94°C for 1 minute, 32 cycles at 94°C for 30 seconds, 61.5°C for 30 seconds, and 72°C for 2 minutes 20 seconds; and one cycle of 72°C for 5 minutes.

tcrβ Genotypes

The *tcrβ* primer set was a three primer set that gave a wildtype product of 1,300 base-pairs and a mutant product of 1,500 base-pairs. The products were amplified using the following PCR protocol: one cycle at 94°C for 1 minute, 32 cycles at 94°C for 30 seconds, 53°C for 30 seconds, and 72°C for 1 minutes 40 seconds; and one cycle of 72°C for 5 minutes.

tcrδ Genotypes

The *tcrδ* primer set was a three primer set that gave a wildtype product of 1,300 base-pairs and a mutant product of 1,500 base-pairs. The products were amplified using the following PCR protocol: one cycle at 94°C for 1 minute, 35 cycles at 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minutes 40 seconds; and one cycle of 72°C for 5 minutes.

Phenotypic Analysis

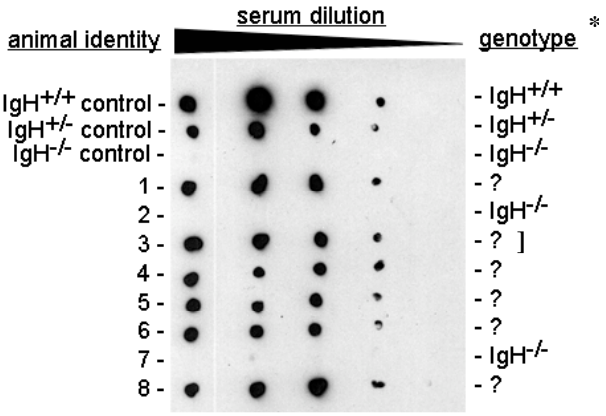
Serum Analysis of *igh* Mice

Peripheral blood (75μL) was collected from the hind leg vein of *igh* colony mice. The blood was microcentrifuged at 4°C for 5 minutes to pellet the red blood cells. The serum supernate was used to blot a nitrocellulose membrane in serial dilutions. Once dry, the nitrocellulose membrane was soaked in Western transfer buffer made up of 250mM Glycine, 25mM Tris-Base, 20% methanol (MeOH) in distilled water. In a rotation oven, the blot was incubated for 15-30 minutes at room temperature in 5% hydrogen peroxide/Western transfer buffer and rinsed 3X with 1X phosphate buffered saline (PBS). The blot was next incubated in a blocker of 20% milk, 0.5% newborn calf serum (NCS), 0.1% TX-100 and 1X PBS for 1 hour at room temperature. The membrane was transferred into a milk solution of 2% milk, 0.2% NCS, 0.1% TX-100, and 1X PBS containing a 1:30,000 dilution of horseradish peroxidase (HRP) conjugated anti-mouse IgG antibody. The membrane was incubated in the antibody for 30 minutes at room temperature and exposed to film after HRP substrates from SuperSignal West Pico Chemiluminescent kit were introduced to the blot (Fig. 9).

FACS Analysis of CIITA

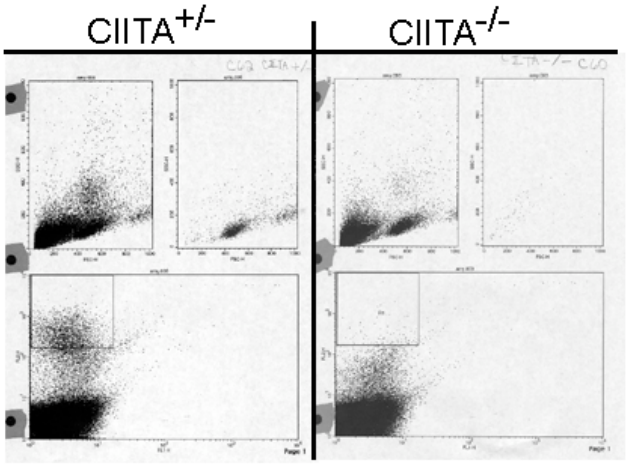
Peripheral blood (75μL) was collected in a heparanized capillary tube from the hind leg vein of *c2ta* colony mice. The blood was mixed with 1mL of 1X PBS with 5μM EDTA. Cells were pelleted in a microcentrifuge at 300xg after 5

Figure 9. Phenotypic Serum Test Analysis of Igh B Cell Mutation



*Heterozygous and wild-type genotypes cannot be inferred phenotypically because igh- is a recessive allele (31).

Figure 10. FACS Analysis of MHC Class II Cell Surface Expression



*Upper left, forward versus side scatter dot plot of peripheral blood FACS analyses; bottom, FL-1 vs. FL-2 dot plot for PBL incubated with anti-MHC class II phytoerythrin (PE)-conjugatd antibody; upper right, forward versus side scatter dot plot of PE-positive PBL.

minutes. The supernatant was discarded, and the cells were resuspended in 500μL of ACK lysis buffer (0.15 M NH4Cl, 1 mM KHCO3, 0.1 mM EDTA, pH 7.3). The lysing process was quenched with 1mL of 1X PBS, and the cells were washed two times in FACS buffer [1X PBS with 1% bovine serum albumin (BSA) and 0.05% sodium azide (NaN_3)]. The cells were then incubated with a 1:60 dilutions of phytoerythrin-(PE-) conjugated anti-mouse MHC class II monoclonal antibody for 1 hour on ice. The cells were washed and resuspended in 200μL of FACS buffer prior to FACS analysis. PE-, MHC class II-positive cells were gated and analyzed (Fig. 10).

Histology

Paraffin Sections

Tissues were fixed in 10% neutral buffered formalin, then processed through an alcohol gradient, 70%, 80%, 95% and 100% ethanol) cleared with xylene and infiltrated with paraffin. After paraffin embedment, 5 μm thick sections were cut on a microtome, dried and then stained with routine hematoxylin and eosin y (H&E).

CHAPTER 3

LABYRINTH MALFORMATION

Introduction

Previous Work from Our Laboratory

In mammals, TBP is composed of two domains: the C-terminal region that is conserved in all eukaryotes and the vertebrate-specific N terminus. The role of the C-terminus in eukaryotic transcription has long been established, but the vertebrate-specific function of the N terminus remains unknown. Our group hypothesized that this conserved vertebrate domain coevolved with transcriptional regulators and basal machinery associated with the invertebrate/vertebrate evolutionary transition. To investigate this hypothesis, a line of mice lacking 111 of the 135 amino acids for the TBP N terminus ($tbp^{\Delta N}$) was generated via targeted mutagenesis. The mutation was expected to alter vertebrate-specific gene pathways that required the TBP N terminus while leaving C-terminal activity intact.

The Vertebrate N Terminus is not Necessary for Basic Cellular Processes. In $tbp^{\Delta N/+}$ intercrosses, the majority of $tbp^{\Delta N/\Delta N}$ fetuses die at midgestation. A logical supposition for this loss was that the fetuses were dying from disruptions in general cellular pathways caused by altered basal transcription. To test this idea, our laboratory created primary mouse embryonic fibroblast (MEF) cell lines from $tbp^{+/+}$, $tbp^{\Delta N/+}$, and

tbp$^{\Delta N/\Delta N}$ fetuses. The cell lines were assayed for genotype differences in proliferation rates, expression of proliferative markers, transcription initiation site fidelity for RNAP II and III, expression of housekeeping genes with either TATA-containing or TATA-less promoters, and mRNA splicing efficiency. No significant differences were identified between the three genotypes, thus suggesting that general eukaryotic cell functions do not require the vertebrate-specifc sequence of the TBP N-terminus or that loss of the N-terminal pathway was readily compensated via alternative pathways (Schmidt, et al., 2003).

Midgestational Lethality of *tbp*$^{\Delta N/\Delta N}$ Fetuses. Fetuses from *tbp*$^{\Delta N/+}$, C57Bl/6 intercrosses were harvested from E8.5 to E13.5. At E8.5 and E9.5, Mendelian ratios of the genotypes were observed, and all fetuses appeared to be developmentally normal. At midgestation (E10.5), though, resorption sites began to appear at a proportional rate to a loss in *tbp*$^{\Delta N/\Delta N}$ fetuses (Hobbs, et al., 2002). By E13.5, this rejection process had ceased leaving only 12% of the *tbp*$^{\Delta N/\Delta N}$ fetuses alive from this point to parturition. Analysis of the surviving mutants revealed that the fetuses had developmentally appropriate organ systems and blood circulation along with a beating heart. However, the majority of the survivors were runted and incapable of surviving to weaning.

tbp$^{\Delta N/\Delta N}$ Mice cannot Genetically Pass on their Survivability to *tbp*$^{\Delta N/\Delta N}$ Offspring. Surprisingly, the few *tbp*$^{\Delta N/\Delta N}$ mice that did survive to adulthood appeared to be healthy and fertile prompting our group to question if this increase in survivability was linked to a heritable genetic characteristic that could be passed from *tbp*$^{\Delta N/\Delta N}$ adults to *tbp*$^{\Delta N/\Delta N}$ offspring. To test this possibility, *tbp*$^{\Delta N/\Delta N}$ males and females were bred to *tbp*$^{\Delta N/+}$ mice, and either pregnant uteri were harvested following the midgestational crisis (E13.5 to E15.5) or litters were allowed to continue to parturition and weaning. Data collected from this experiment showed that survival rates for homozygous mutants did not increase at midgestation or at weaning, thus, suggesting that factors affecting *tbp*$^{\Delta N/\Delta N}$ survival were not associated with heritable genetic traits.

tbp$^{\Delta N/\Delta N}$ Fetuses are Lost as a Result of Defective Placentas. When no apparent defects were observed in *tbp*$^{\Delta N/\Delta N}$ fetuses, we began to focus our attention toward the placenta. The placenta is a transient organ that controls how maternal and the embryonic tissue interacts. Placental tissues provide a transport surface for oxygen and nutrients, control the maternal endocrine system during placental vasogenesis, and foster maternal immune tolerance during pregnancy (Cross, et al., 2002a; Hemberger and Cross, 2001). Alterations in pathways involved in placental development or maintenance result in runted fetuses or fetal death (Hemberger and Cross, 2001). Therefore, we

wanted to determine if the death of *tbp*$^{\Delta N/\Delta N}$ murine fetuses also coincided with a placental defect(s).

Preliminary histological analysis of *tbp*$^{+/+}$ and *tbp*$^{\Delta N/\Delta N}$ placentas revealed that mutant but not wildtype placentas at E10.5 showed signs of hemorrhaging with the occasional visible mixing of nucleated fetal red blood cells (RBCs) and maternal RBCs. At E12.5 and later, massive tissue destruction was also a prevalant feature of mutant but not wildtype placental histology. To verify that the mutant placental pathology was due to the loss of TBP N terminal activity in the placenta, our laboratory generated *tbp*$^{\Delta N/\Delta N}$ fetuses with *tbp*$^{+/+}$ tetraploid placentas using chimeric fusion techniques. These *tbp*$^{\Delta N/\Delta N}$/ *tbp*$^{+/+}$ hybrids survived the midgestational crisis, thus, bolstering the idea that *tbp*$^{\Delta N/\Delta N}$ fetuses die at midgestation from placental defects (Hobbs, et al., 2002).

Results/Discussion

Defects Arise in *tbp*$^{\Delta N/\Delta N}$ Placentas between E8.5 and E9.5

Previous histological analysis of wildtype and mutant placentas suggested gross morphological defects that were in *tbp*$^{\Delta N/\Delta N}$ placental tissue but not *tbp*$^{+/+}$ tissue (Hobbs, et al., 2002). The widespread tissue destruction and hemorrhages observed between E10.5 and E13.5 suggested that a more subtle, mutant phenotype might be found at an earlier stage of placental development. Thus, earlier placentas were examined by crossing *tbp*$^{\Delta N/+}$ males with *tbp*$^{\Delta N/+}$ females, harvesting the pregnant uteri from E8.5 to E12.5, determining the *tbp*

genotype for the fetuses via PCR, and performing histological analysis on the recovered placentas. The goal was to document the progression of morphological changes in *tbp*$^{\Delta N/\Delta N}$ placentas by comparing mutant placentas to *tbp*$^{+/+}$ littermate controls (Fig. 11). This series showed that the differences between wildtype and mutant placentas were visible as early as E8.5. At this early stage, the *tbp*$^{\Delta N/\Delta N}$ placentas had all four tissue layers, but, the thickness and cellularity of the spongiotrophoblast and labyrinth layers were greatly diminished. At E9.5, all of the placental layers were still present and the spongiotrophoblast and labyrinth regions were reduced; however, the spongiotrophoblast layer was losing its characteristic cellular features and its structural integrity, while the labyrinth region was composed of a condensed layer of chorion-like cells. By E10.5, mature, highly vascularized placentas were found at *tbp*$^{+/+}$ concepti, whereas hemorrhage sites were present in the spongiotrophoblast regions and maternal decidua of *tbp*$^{\Delta N/\Delta N}$ placentas. The labyrinth layers of these mutant placentas also remained a condensed, avascular, chorion-like cell layer. Finally, E11.5 and E12.5 *tbp*$^{\Delta N/\Delta N}$ placentas showed defects that were similar to E10.5 placentas; however, hemorrhages were more frequent at these stages. Few E12.5 *tbp*$^{\Delta N/\Delta N}$ fetuses and placentas could be examined because most had already been resorbed (data not shown).

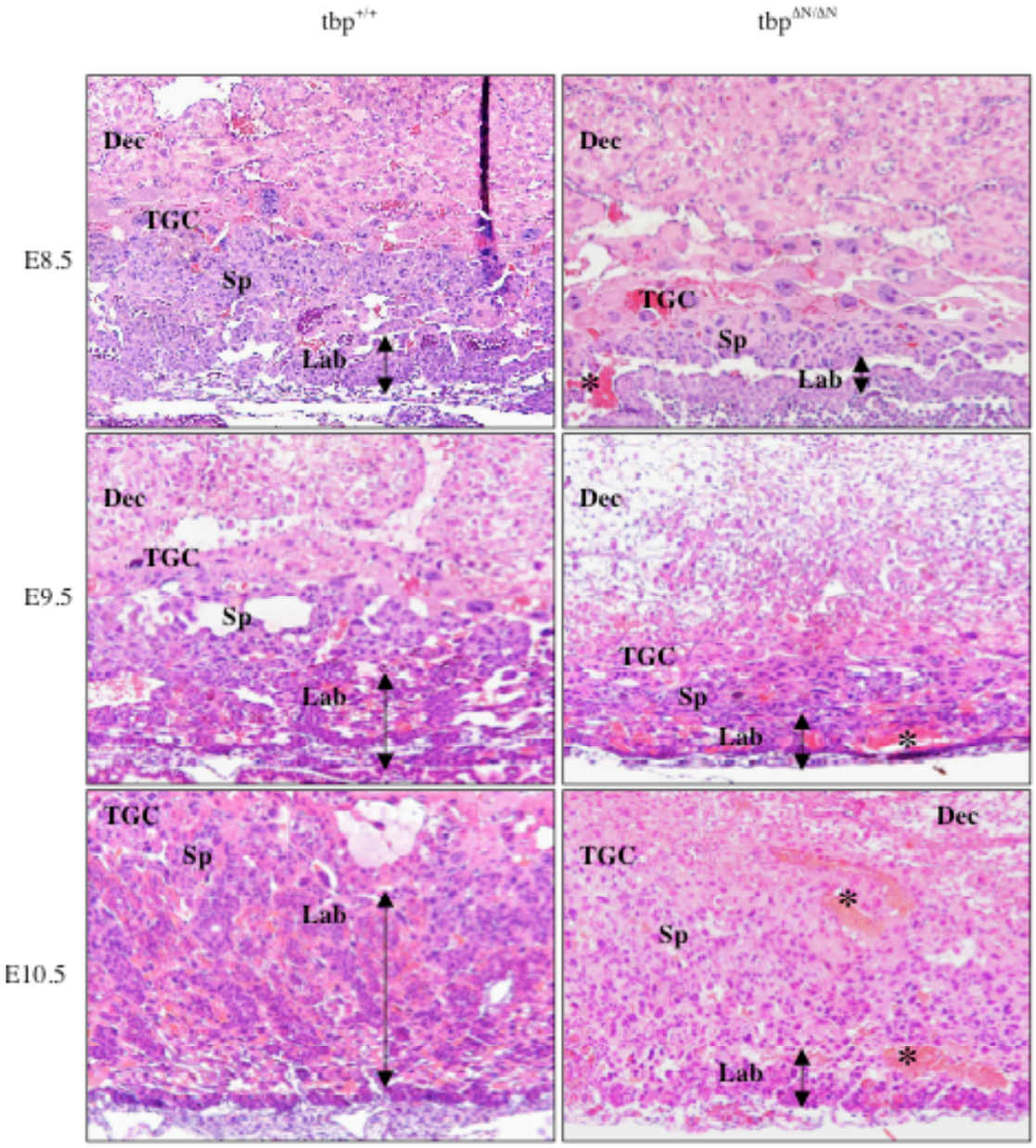
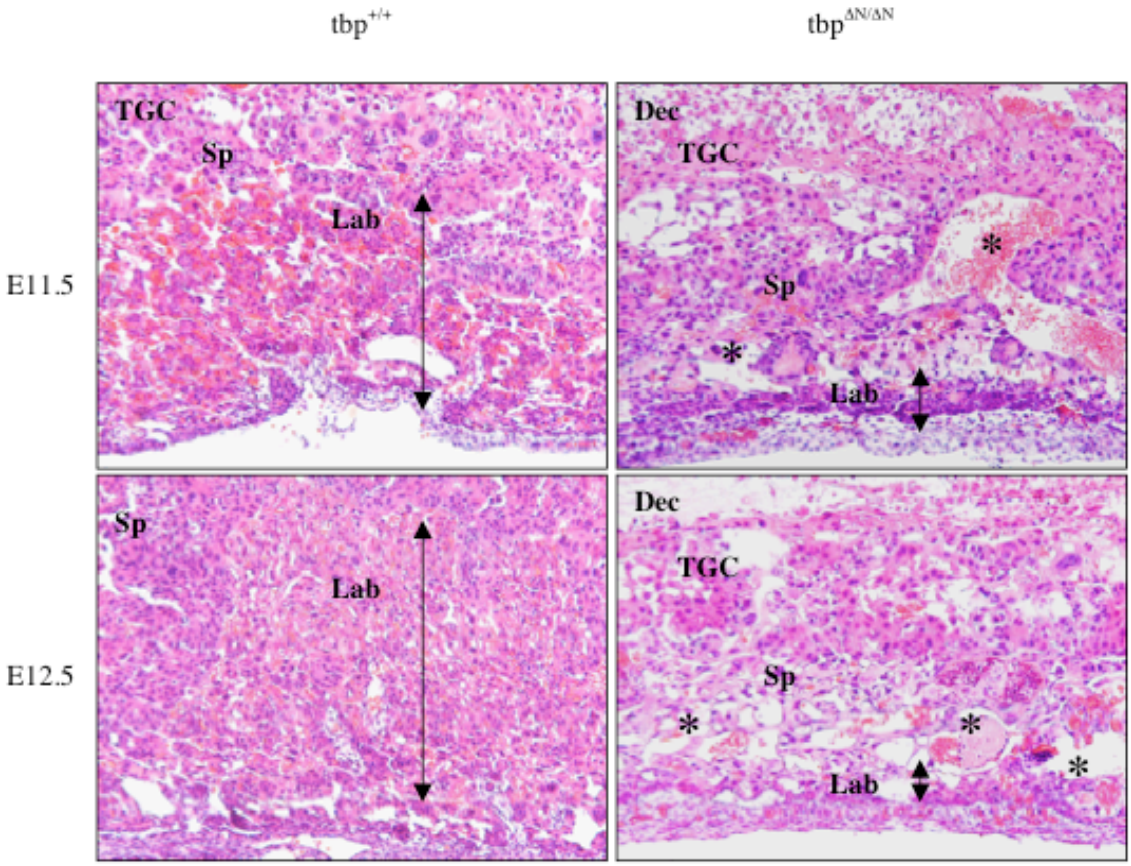


Figure 11. Histological anlaysis of changes in *tbp*$^{+/+}$ and *tbp*$^{\Delta N/\Delta N}$ placentas from E8.5 to E12.5. An arrow designates the width of the labyrinth region and asterisks are used to identify hemorrhages and placental degradation.

Figure 11. (continued)



Labyrinths of *tbp*$^{\Delta N/\Delta N}$ Placentas are Malformed

A large number of transgenic mouse lines have homozygous mutant fetusus that die at midgestation from placental defects. These defects are often associated with problems in labyrinth genesis, a process which begins at E7.5 and is completed by E9.5 (Hemberger and Cross, 2001). Starting at E8.5, we found that the labyrinth layer of *tbp*$^{\Delta N/\Delta N}$ fetuses was smaller than that of the *tbp*$^{+/+}$ fetuses (Fig. 11). Also, the *tbp*$^{\Delta N/\Delta N}$ labyrinth showed little or no sign of vascular development at E8.5 and E9.5, key time points for vessel formation

(Muntener and Hsu, 1977). Therefore, we theorized that the *tbp*$^{\Delta N}$ altered a TBP N terminus-specific pathway in the *tbp*$^{\Delta N/\Delta N}$ placental labyrinth and that this labyrinth defect was associated with the *tbp*$^{\Delta N/\Delta N}$ rejection pathway in immunocompetent dams.

To test this, we bred *tbp*$^{\Delta N/+}$ male mice with tbp$^{\Delta N/+}$; rag1$^{-/-}$ female mice, harvested the pregnant uteri at E9.5, determined the genotypes of the *tbp* fetuses, and analyzed the labyrinth region of the *tbp*$^{+/+}$ and *tbp*$^{\Delta N/\Delta N}$ placentas from those matings (Fig. 12). At E9.5, the rescued *tbp*$^{\Delta N/\Delta N}$ fetuses had intact placental labyrinths with nucleated fetal RBCs infiltrating into the tissue layer as should be seen during normal development. To see nucleated RBCs in the labyrinth interior required that the first stage of labyrinth genesis, chorio-allantoic fusion, was occuring in the *tbp*$^{\Delta N/\Delta N}$ placentas (Muntener and Hsu, 1977; Schreiber, et al., 2000). Also, rescued fetuses had as large if not larger labyrinth widths than their *tbp*$^{+/+}$ littermates (Fig. 12), where as the previous findings in immunocompetent dams showed that *tbp*$^{\Delta N/\Delta N}$ placentas had much smaller labyrinth regions (Fig. 11). This suggested that if the *tbp*$^{\Delta N/\Delta N}$ placenta was triggering an immune response, then the rejection response was likely blocking continued development of the *tbp*$^{\Delta N/\Delta N}$ labyrinth in immunocompotent dams.

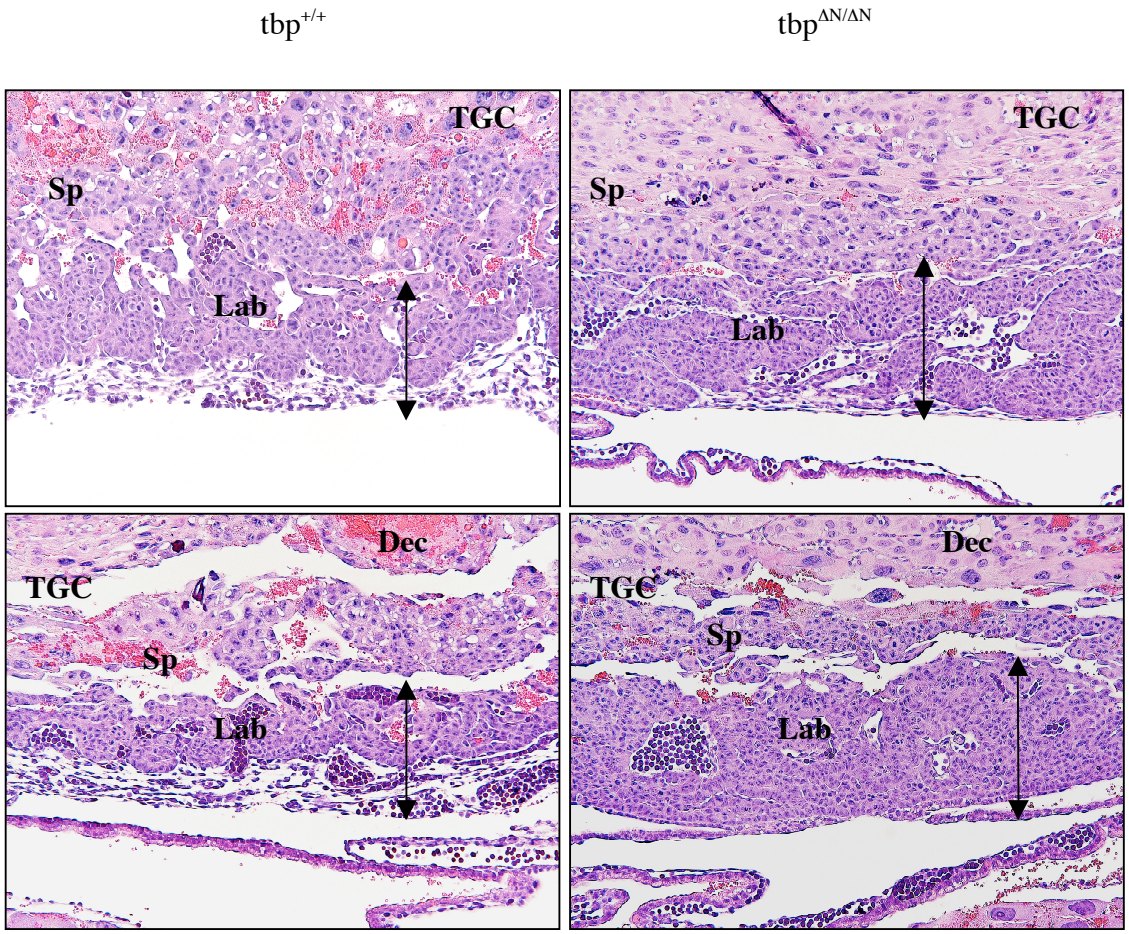
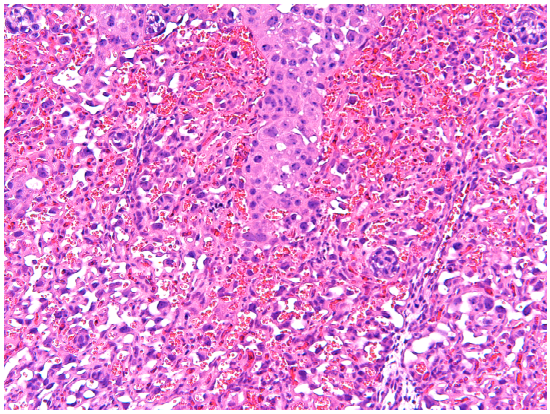


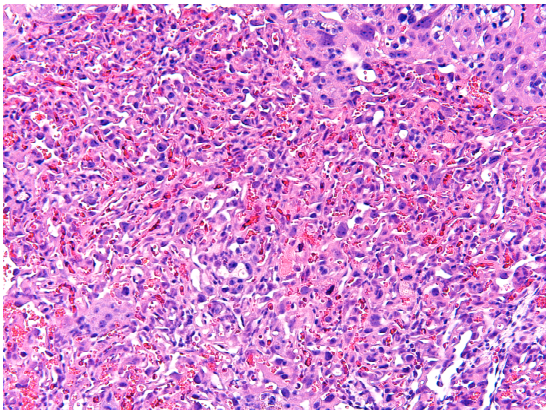
Figure 11. Histological analysis of E9.5 *tbp*$^{+/+}$ and *tbp*$^{\Delta N/\Delta N}$ placentas in *tbp*$^{\Delta N/\Delta N}$ rescuing *rag1*$^{-/-}$ dams. The arrows designate the width of the labyrinth region. Mutant and wildtype placentas are paired horizontally beside their littermate.

The *tbp*$^{\Delta N/\Delta N}$ Labyrinth has Decreased Vascularization. The placental labyrinths of the rescued *tbp*$^{\Delta N/\Delta N}$ fetuses, though similar, did not have the same phenotype as *tbp*$^{+/+}$ labyrinths (Fig. 12). The chorion-derived cells in the rescued mutant placentas seemed to segregate into thick clumps around the newly formed blood

vessels reminiscent of the clustered chorion-like layer found in the placental tissue from E9.5 *tbp*$^{\Delta N/\Delta N}$ fetuses in immunocompetent mothers (Fig. 11). Also, the number of trophoblast-lined blood vessels within the labyrinth of the E9.5 rescued fetuses appeared to be fewer than those in tbp$^{+/+}$ fetuses from the same dams (Fig. 12). To verify if this decreased vascularization real phenotypic difference between wildtypes and mutants, *tbp*$^{\Delta N/+}$ male mice were bred with tbp$^{\Delta N/+}$; rag1$^{-/-}$ female mice where the pregnant uteri were harvested between E13.5 and E14.5 (following any possible changes due to midgestation events). The genotypes of the *tbp* fetuses were determined, and the labyrinth region of the *tbp*$^{+/+}$ and *tbp*$^{\Delta N/\Delta N}$ placentas were analyzed using morphometric analysis techniques (Fig. 13). The data revealed that the *tbp* homozygous mutants had approximately 2-fold less blood vessel area within the labyrinth as *tbp* wildtype fetuses. This new data led to the conclusion that the *tbp*$^{\Delta N}$ was involved in labyrinth vascular development and that loss of signals regulating the process was most likely initiating the maternal immune rejection response.



$tbp^{+/+}$



$tbp^{\Delta N/\Delta N}$

Fetal *tbp* Genotypes	Average Pixels (*10^3)	Average Length (*10^3cm)
+/+	622	22.0
ΔN/ΔN	330	11.7

Figure 13. Morphometric analysis of E14.5 placental vasculature from *rag1*$^{-/-}$ females

Conclusions

Loss of the TBP N Terminus Leads to Rejection Due to Placental Labyrinth Malformations

Early analysis of the *tbp*$^{\Delta N/\Delta N}$ placental defect showed that *tbp*$^{\Delta N/\Delta N}$ fetuses could survive in mothers from various immunocompromised mouse lines (i. e. SCID)(Hobbs, et al., 2002). This suggested that the TBP N terminus was playing a role in an immune regulatory pathway which allowed trophoblast cells to control maternal immune responses. However, the evidence presented here suggests that the N terminus of TBP may not be involved in an immune regulatory pathway, rather it appears to control labyrinth vascularization within the placenta. In immunocompotent mothers, labyrinth malformation in *tbp*$^{\Delta N/\Delta N}$ placentas

appears to coincide with midgestational abortion, whereas in severely immunocompromised mothers, a vascular defect still occurs but the mother cannot mount a rejection response against the abnormal vasculature. The labyrinth cell population continues to expand, creating a vascular network that has substantially less surface area for nutrient and gaseuos exchange while still meeting the very minimum nutrional requirements for fetal development. This may explain why unpublished data collected by Nicole Hobbs and Jean Kundert in our laboratory shows that $tbp^{\Delta N/\Delta N}$ mice, which survive to birth, are approximately 30% smaller than their $tbp^{+/+}$ littermates.

The TBP N Terminus May Regulate
Pathways in Chorion-derived Cells .

Currently, no TBP N-terminal target genes have been identified with respect to placental development or labyrinth vascularization pathways. We may, however, have an idea of what placental cell line utilizes the TBP N terminal pathway. The labyrinth is composed of two cells types, the chorion- and the allantoic-derived cells (Muntener and Hsu, 1977). Chorion cells arise from a trophectoderm-derived lineage and differentiate into labyrinth trophoblast cells that reside in the non-lumen space between vascular endothelial cells (Papaioannou, 1982). These chorion-derived cells are believed to regulate nutrient exhange and waste management in the mature placenta (Anson-Cartwright, et al., 2000; Schreiber, et al. 2000). Allantoic-derived cells arise from the primitive streak and are the endothelial-like trophoblast cells that line both

maternal and fetal blood vessels in the labyrinth (Papaioannou, 1982). These cells form the surface where nutrients, oxygen, and waste are exchanged (Leiser and Kaufmann, 1994; Wooding and Flint; 1994). In previous experiments using diploid/tetraploid morula fusion techniques, $tbp^{\Delta N/\Delta N}$ fetuses were rescued when attached to placentas with $tbp^{+/+}$ trophoblast cells (Hobbs, et al. 2002). However, in the fusion experiments, the allantois would arise from the $tbp^{\Delta N/\Delta N}$ embryo-derived cells from the primitive streak leaving the chorion as the only source of $tbp^{+/+}$ cells in the labyrinth. Along with the histological data that connects the $tbp^{\Delta N/\Delta N}$ labyrinth phenotype with chorionic cell clustering, this data suggest that chorionic cells require the N terminus of TBP for normal development.

CHAPTER 4

REJECTION-RELATED IMMUNE RESPONSES

Introduction

As the placenta matures, maternal cells and blood gain access to trophoblast cells and fetal antigens. Such contact is dramatically increased during labyrinth genesis (E8.5-E10.5) as the expanding vascular network creates more surface area between the fetus and the mother. Normally, this exposure does not elicit a significant maternal immune response; however, in mouse models with placental abnormalities or aberrant fetal antigen recognition, maternal immune cells can attack the conceptus causing fetal rejection or intrauterine growth restriction (Aoki, et al., 2007; Cross, et al. 2006; Singh and Verma, 1987).

Previous Work from Our Laboratory

tbp$^{\Delta N/\Delta N}$ Fetuses are Rescued in Immunocompromised Mothers

Genetic complementation of *tbp*$^{\Delta N}$ with SCID and *rag-1* Null Alleles

. Because the loss of *tbp*$^{\Delta N/\Delta N}$ fetuses was found to occur around E10.5 in association with a placental defect, it was important to determine if the *tbp*$^{\Delta N}$-related abnormality was triggering a maternal immune response that rejects *tbp*$^{\Delta N/\Delta N}$ concepti (Hobbs, et al., 2002). To examine this possibility, *tbp*$^{\Delta N}$ mice were bred to

immunocompromised animals who either had Severe Combined Immunodeficiency (SCID) or harbored null alleles for *rag-1*. From this, *tbp*$^{\Delta N/+}$ immunodeficient female mice were generated, which were mated to immune-wildtype male mice. If *tbp*$^{\Delta N/\Delta N}$ pups survived past midgestation in dams incapable of mounting an immune response, it would suggest that *tbp*$^{\Delta N/\Delta N}$ fetal

Table 1. rag1 Rescue of tbp$^{\Delta N/\Delta N}$ Fetuses Past Mid-gestation

Parental Genotypes		**Fetal Harvests (16.5±3 d.p.c)***		
Paternal	**Maternal**	**Observed tbp Ratios +/+ : ΔN/+ : ΔN/ΔN**	**Mendelian tbp Ratios +/+ : ΔN/+ : ΔN/ΔN**	**Percent Survival tbp$^{\Delta N/\Delta N}$**
tbp$^{\Delta N/+}$; rag1$^{+/+}$	tbp$^{\Delta N/+}$; rag1$^{-/-}$	22 : 43 : 20	22 : 44 : 22	91%‡

*All fetuses are rag$^{+/-}$.

‡The tbp genotype ratio from fetal harvests (11 pregnancies) of tbp$^{\Delta N/+}$; rag1$^{-/-}$ dams is not significantly different from a Mendelian 1:2:1 ratio (α=0.05, 0.05>p>0.025). Data set is a summary of published (see Reference 1) and unpublished results (provided by Nicole Hobbs).

Table 2. Survival Rate of Tbp$^{\Delta N/\Delta N}$ Fetuses as a Function of Fetal β_2m Genotype

Fetal Genotype	**tbp**			**Percent Survival tbp$^{\Delta N/\Delta N}$‡**
β_2m	**+/+**	**ΔN/+**	**ΔN/ΔN**	
+/+	25	42	2	**8%**
-/-	29	55	14	**48%** (p<0.001)‡

‡ Chi-square test that the tbp genotype ratios of β_2m$^{-/-}$ are significantly different from those of β_2m$^{+/+}$ fetuses, α=0.05 (1).

loss in immune-normal dams was immune mediated.

Upon harvesting these litters between E13.5 and E15.5, it was found that survival rates for *tbp*$^{\Delta N/\Delta N}$ fetuses were approximately 4-fold higher in SCID mothers and complete rescue was attained in *rag-1*$^{-/-}$ mothers (as compared to the immune wildtype controls)(Table 1)(Hobbs, et al., 2002). This data supported the hypothesis that *tbp*$^{\Delta N/\Delta N}$ fetuses were likely dying at midgestation due to maternal immune responses.

β_2M May Play a Role in Activating Maternal Immune Responses. Since the *tbp*$^{\Delta N/\Delta N}$ fetuses were rescued by immunocompromised dams and *tbp*$^{\Delta N/\Delta N}$ placentas were defective in immune wildtype dams, it was necessary to learn more about the placental factors that might be stimulating the maternal immune system and initiating rejection (Hobbs, et al., 2002).

Trophoblast cells regulate maternal immune responses. Some trophoblast cells can activate immune-suppressive pathways (i. e. Crry and IDO) (Xu, et al., 2000, Munn, et al.; 1998). Other trophoblast cells down-regulate pathways involved in antigen presentation. For example, endothelial-like trophoblast cells that line the labyrinth vasculature down-regulate their expression of classical MHC I. This, in turn, decreases fetal antigen presentation and “hides” the trophoblast cells from immune cells such as cytotoxic T cells (Ait-Azzouzene, et al., 2001; Jaffe, et al., 1990; Morello, et al., 1985).

Since trophoblast cells regulate antigen presentation at the maternal/fetal interface as a means to deter immune responses, it was surmised that *tbp*$^{\Delta N}$ was triggering inappropriate antigen presentation at the interface in homozygous mutant placentas thus leading to maternal attack of placental tissue. To investigate this hypothesis, the *tbp*$^{\Delta N}$ mouse line was bred with β_2*m*- deficient mice to generate females that were *tbp*$^{\Delta N/+}$; $\beta_2 m^{+/-}$ and males that were either *tbp*$^{\Delta N/+}$; $\beta_2 m^{+/+}$, *tbp*$^{\Delta N/+}$; $\beta_2 m^{+/-}$, or *tbp*$^{\Delta N/+}$; $\beta_2 m^{-/-}$. Crosses of these males and females revealed that *tbp*$^{\Delta N/\Delta N}$; $\beta_2 m^{-/-}$ fetuses had a greater than 6-fold increase in survival at midgestation as compared to *tbp*$^{\Delta N/\Delta N}$; $\beta_2 m^{+/+}$ fetuses (Table 2). This data suggested that, at midgestation, a β_2*m*-dependent process was involved in maternal recognition of the *tbp*$^{\Delta N/\Delta N}$ placenta and fetus (Hobbs, et al. 2002).

tbp$^{\Delta N/\Delta N}$ Rejection Does Not Establish Immunological Memory. Classical MHC I immune responses initiate adaptive immune pathways that eventually establish immunological memory. Memory responses allow the immune system to more quickly and accurately address previously encountered antigens (Altman, et al., 1996; Bousso and Kourilsky, 1999; McHeyzer-Williams and Davis, 1995). Therefore, if *tbp*$^{\Delta N/\Delta N}$ fetuses are rejected via a classical MHC I adaptive immune response, then recognition of the homozygous mutant placental/fetal antigens in the first pregnancy would initiate a memory response and a dam should become less and less tolerant of *tbp*$^{\Delta N/\Delta N}$ antigens in each consecutive pregnancy following the first one. To determine if memory was being established in dams

where *tbp*$^{\Delta N/\Delta N}$ fetuses were being rejected, *tbp*$^{\Delta N/+}$ males and females were crossed where the females had presumably been exposed to *tbp*$^{\Delta N/\Delta N}$ placental/fetal antigens during previous pregnancies. These dams were harvested between E13.5 and E15.5, and the fetuses were genotyped for *tbp.* Results indicated that *tbp*$^{\Delta N/\Delta N}$ were not rejected at a greater rate with consecutive exposure to *tbp*$^{\Delta N/\Delta N}$ fetal antigens. This data was further supported by husbandry records which showed that *tbp*$^{\Delta N/\Delta N}$ adults had equally come from naïve and experienced dams. Hence, *tbp*$^{\Delta N/\Delta N}$ fetuses were, most likely, not being rejected via the classical MHC I/antigen presentation pathway or its associated adaptive immune responses since immunological memory was not being establish during the abortive process.

Results/Discussion

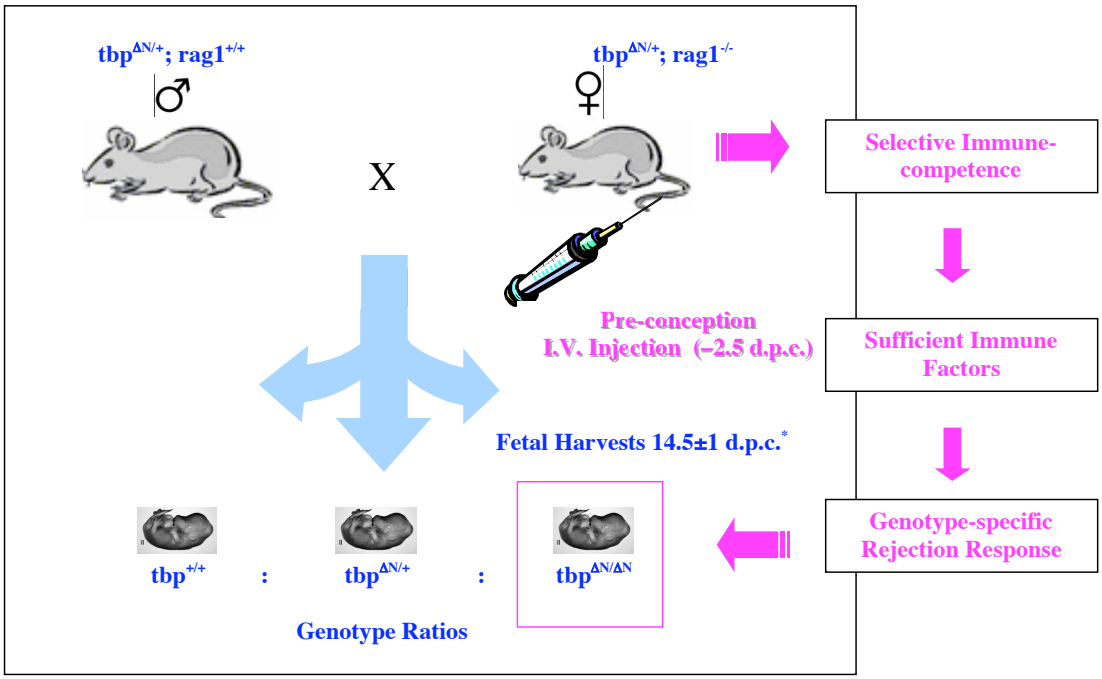
Rejection can be Established in *rag1*-Deficient Dams

Midgestational rescue of *tbp*$^{\Delta N/\Delta N}$ fetuses in *tbp*$^{\Delta N/+}$; *rag1*$^{-/-}$ mothers suggested maternal immune factors respond to the *tbp*$^{\Delta N/\Delta N}$ placenta (Table 1). It was important to determine whether or not a *tbp*$^{\Delta N/\Delta N}$-specific rejection response could mounted in these mothers through immune reconstitution.

To test this, spleens from immune-wildtype mice were harvested, crushed, and injected into *tbp*$^{\Delta N/+}$; *rag1*$^{-/-}$ dams 2.5 days prior to conception (Fig. 14). Subsequent matings with *tbp*$^{\Delta N/+}$*; rag1*$^{+/+}$ males resulted in ~82% loss of tbp$^{\Delta N/\Delta N}$ fetuses at midgestation (Fig.14; Table 3), indicating that the spleen immune

factors were sufficient for rejection. In control experiments, transfers of *rag1*$^{-/-}$ crushed spleens into *tbp*$^{\Delta N/+}$*; rag1*$^{-/-}$ dams showed only 14% loss of tbp$^{\Delta N/\Delta N}$ fetuses (Table 3), confirming that it was immune factors present in the immunocompetent spleen and not the procedural manipulation that caused genotype-specific loss of the mutant fetuses. This indicated that maternal immune factors, which are present in normal spleen, are adequate for rejecting *tbp*$^{\Delta N/\Delta N}$ fetuses.

Figure 14. Establishment of tbp$^{\Delta N/\Delta N}$ Fetal Rejection in rag1$^{-/-}$ Dams



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*All fetuses are rag1$^{+/-}$.

Table 3. Preconception Adoptive Transfer into Immune-compromised Dams

Parental Genotypes		Fetal Harvests (15.5±2 d.p.c)				
		Injected Spleen Source	Number of Litters	Observed tbp Ratios +/+ : ΔN/+ :ΔN/ΔN	Mendelian tbp Ratios +/+ : ΔN/+ : ΔN/ΔN	Percent Survival $tbp^{\Delta N/\Delta N}$
Paternal	Maternal					
$tbp^{\Delta N/+}$; $rag1^{+/+}$	$tbp^{\Delta N/+}$; $rag1^{-/-}$	$tbp^{\Delta N/+}$; $rag1^{+/+}$	11	27 : 41 : 5	27 : 54 : 27	**19%**‡
$tbp^{\Delta N/+}$; $rag1^{+/+}$	$tbp^{\Delta N/+}$; $rag1^{-/-}$	$tbp^{+/+}$; $rag1^{-/-}$	4	7 : 16 : 6	7 : 14 : 7	86%*

‡Chi-square test that tbp genotype ratios of fetuses from injected $tbp^{\Delta N/+}$; $rag1^{-/-}$ dams are significantly different from those of immune-wild-type dams, α=0.05. All fetuses are $rag1^{+/-}$ and are functionally immune-wild-type.
*The genotype ratio from dams injected with the $tbp^{+/+}$; $rag1^{-/-}$ spleen source are not statistically significant from a Mendelian 1:2:1 wild-type to heterozygous to homozygous ratio.

Fetal Rejection is not Caused by a Single Adaptive Immune Function .

To determine which cellular components of the maternal adaptive immune system may be necessary for this rejection response, the *tbp*$^{\Delta N}$ mutation was bred into various transgenenic mouse lines with adaptive immune system defects as well as injected dams from *tbp*$^{\Delta N/+}$ crosses with immune cell-depleting antibodies. Fetal genotypes were then collected from *tbp*$^{\Delta N/+}$ crosses where the sire was immune-wildtype and the dam was homozygous mutant for a given adaptive immune defect (Table 4). Midgestational survival of *tbp*$^{\Delta N/\Delta N}$ fetuses from these harvests suggested that the maternal adaptive immune responses could be divided into three distinct categories, denoted here as Group I (yellow), II (green), and III (red). Group I mice were the immune-wildtype controls as well as lines with no significant change in survivability from immunocompetent dams which included the B cell-null (*igh*$^{-/-}$)(Kitamura, et al., 1991), major

histocompatibility class II (MHC II) transactivator-null (*c2ta*$^{-/-}$)(Chang, et al., 1989), and αβ T cell receptor-deficient (*tcrβ*$^{-/-}$) mice (Mombaerts, et al., 1992). Group II was comprised of β_2*m*$^{-/-}$ and *rag1*$^{-/-}$ dams that have a greater than two-fold increase in the survival of *tbp*$^{\Delta N/\Delta N}$ fetuses, and Group III dams had a 2-fold or more decrease in survival of mutants to midgestation. Group III included mice that lack $CD8^+$ cells (*cd8*$^{-/-}$)(Fung-Leung, et al., 1991), interferon-γ receptors (*ifgr*$^{-/-}$)(Huang, et al., 1993), γδ Tcells (*tcrδ*$^{-/-}$), or all T cells subsets (*tcrβ*$^{-/-}$**;** *tcrδ*$^{-/-}$)(Itohara, et al., 1993).

Group I data implied that humoral immunity, maternal MHC class II/ antigen presentation, and αβ T cells by themselves have little or no involvement in the rejection process.

Group II showed that the loss of β_2M-dependent processes from either the mother or the fetus (as seen in previous experiments) could lead to mother or the fetus (as seen in previous experiments) could lead to partial rescue of *tbp*$^{\Delta N/\Delta N}$ fetuses. However, because Group III showed that *cd8*$^{-/-}$ dams exascerbated the rejection response, this suggested that maternal-derived β_2M processes were most likely not involved in classical MHC I/fetal antigen presentation processes during rejection.

Group III mothers such as *cd8*$^{-/-}$ dams revealed that the loss of certain adaptive immune functions could cause an increase in *tbp*$^{\Delta N/\Delta N}$ fetal rejection. This suggested that regulatory pathways were most likely still intact within pregnant uteri, and these regulatory pathways could be directly antagonizing the

Parental Genotypes		Fetal Harvests (14.5±1 d.p.c)			
Paternal	**Maternal**	**Number of Litters**	**Observed tbp Ratios +/+ : ΔN/+ : ΔN/ΔN**	**Mendelian tbp Ratios +/+ : ΔN/+ : ΔN/ΔN**	**Percent Survival $tbp^{\Delta N/\Delta N\ddagger}$**
$tbp^{\Delta N/+}$	$tbp^{\Delta N/+}$	29	61 : 111 : 7	61 : 122 : 61	**12%**
$tbp^{\Delta N/+}$; $igh^{+/+}$	$tbp^{\Delta N/+}$; $igh^{-/-}$	13	27 : 58 : 3	27 : 54 : 27	**11%**
$tbp^{\Delta N/+}$; $c2ta^{+/+}$	$tbp^{\Delta N/+}$; $c2ta^{-/-}$	19	38 : 76 : 6	38 : 76 : 38	**16%**
$tbp^{\Delta N/+}$; $tc\beta^{+/+}$	$tbp^{\Delta N/+}$; $tc\beta^{-/-}$	14	19 : 42 : 3	19 : 38 : 19	**16%**
$tbp^{\Delta N/+}$; $\beta_2m^{+/+}$	$tbp^{\Delta N/+}$; $\beta_2m^{-/-}$	22	42 : 85 : 14	42 : 84 : 42	**33%**
$tbp^{\Delta N/+}$; $rag1^{+/+}$	$tbp^{\Delta N/+}$; $rag1^{-/-}$	11	22 : 43 : 20	22 : 44 : 22	**91%**
$tbp^{\Delta N/+}$; $cd8^{+/+}$	$tbp^{\Delta N/+}$; $cd8^{-/-}$	11	16 : 22 : 1	16 : 32 : 16	**6%**
$tbp^{\Delta N/+}$; $ifgr^{+/+}$	$tbp^{\Delta N/+}$; $ifgr^{-/-}$	15	37 : 46 : 1	37 : 74 : 37	**3%**
$tbp^{\Delta N/+}$; $tcr\delta^{+/+}$	$tbp^{\Delta N/+}$; $tcr\delta^{-/-}$	19	34 : 86 : 0	34 : 68 : 34	**0%**
$tbp^{\Delta N/+}$; $tcr\delta^{+/+}$: $tc\beta^{+/+}$	$tbp^{\Delta N/+}$; $tcr\delta^{-/-}$: $tc\beta^{-/-}$	12	25 : 29 : 0	25: 50 : 25	**0%**

Table 4. Survival rate of *tbp*$^{\Delta N/\Delta N}$ past midgestation in dams with various immunocompromised backgrounds

abortive processes or maybe part of a control network to maintain the rest of a litter when one of the fetuses is aborted.

Finally, antibody depletion studies confirmed both *c2ta*- and *cd8*-null breeding data as dams depleted of $CD4^+$ or $CD8^+$ cells also did not show an increase in *tbp*$^{\Delta N/\Delta N}$ survivability (Table 6).

None of the new mutant mouse lines tested or antibody depletion studies, however, correlated with complete rescue of *tbp*$^{\Delta N/\Delta N}$ fetus leading us to conclude that a single adaptive immune pathway is not responsible for the abortion of *tbp* homozygous fetuses at midgestation.

Maternal Adaptive Immune Cells do not Change in Response to *tbp*$^{\Delta N/\Delta N}$ Rejection

tbp$^{\Delta N/\Delta N}$ Rejection does not Alter Para-aortic Lymph Nodes Lymphocyte Populations. In immunocompetent dams at E9.5, tissue destruction and random hemorrhaging was visible in *tbp*$^{\Delta N/\Delta N}$ placentas. However, by histological analysis maternal lymphocytes did not appear to be infiltrating into mutant tissue. Thus, we wanted to verify if maternal adaptive immune cells were mounting a response against the *tbp*$^{\Delta N/\Delta N}$ fetuses. This was tested by breeding *tbp*$^{\Delta N/+}$ males and females and *tbp*$^{+/+}$ males and females and harvesting pregnant uteri along with spleens, thymuses, and uterine tissue-draining para-aortic lymph nodes (as established in Gottesman and Stutman, 1980) from dams at E8.5 and E9.5 (Fig. 15). Fetuses were genotyped for *tbp* to confirm that the appropriate dams were carrying *tbp*$^{\Delta N/\Delta N}$ fetuses, and immune cells were isolated from the spleen, thymus, and the lymph nodes of each dam for treatment with various anti-CD antibodies and FACS analyzed. Our results indicated no significant change in the absolute number of B cells, total T cells, $\alpha\beta$ T cells, $\gamma\delta$ T cells, CD4$^+$ $\alpha\beta$ T cells, CD8$^+$ $\alpha\beta$ T cells, and NKT cells was seen. This, along with previous experiments where para-aortic lymph

nodes during later stages (E11.5-E13.5) of pregnancy were analyzed (data not shown), suggested that adaptive immune cells in the lymph nodes were not responding to *tbp*$^{\Delta N/\Delta N}$ fetal/placental defects.

Conclusions

Because *tbp*$^{\Delta N/\Delta N}$ fetuses are rescued in *rag1*-deficient and SCID dams and an increase in midgestational survival is associated with the loss of fetal-derived β_2M expression in the placenta, our laboratory has focused on determining which maternal adaptive immune cell population is required for rejection. Analysis of *tbp*$^{\Delta N}$ in various immunodeficient mouse lines or *tbp*$^{\Delta N/+}$ crosses with immune cell-depleted dams revealed that a single cell type was not necessary for rejecting mutant fetuses (Table 4). However, immune wildtype splenic cells caused mutant-specific rejection when the organ was ground up and injected into *rag1*$^{-/-}$ dams (Table 3). This showed that a mixture of splenic cells were sufficient for transforming *rag1*$^{-/-}$ dams into abortion prone mothers, but that the loss of a single adaptive immune cell population could not prevent *tbp*$^{\Delta N/\Delta N}$ midgestational rejection. From these observations, three possible conclusions were reached: 1) multiple adaptive immune cell subsets can initiate a rejection response, 2) A genetic defect, which prevents an immune cell population from forming or functioning, may lead to the development of a compensatory mechanism that replaces the immune activity lost (Kaufmann and Ladel, 1994), or 3) the adaptive immune system does not initiate the rejection process, rather components of the

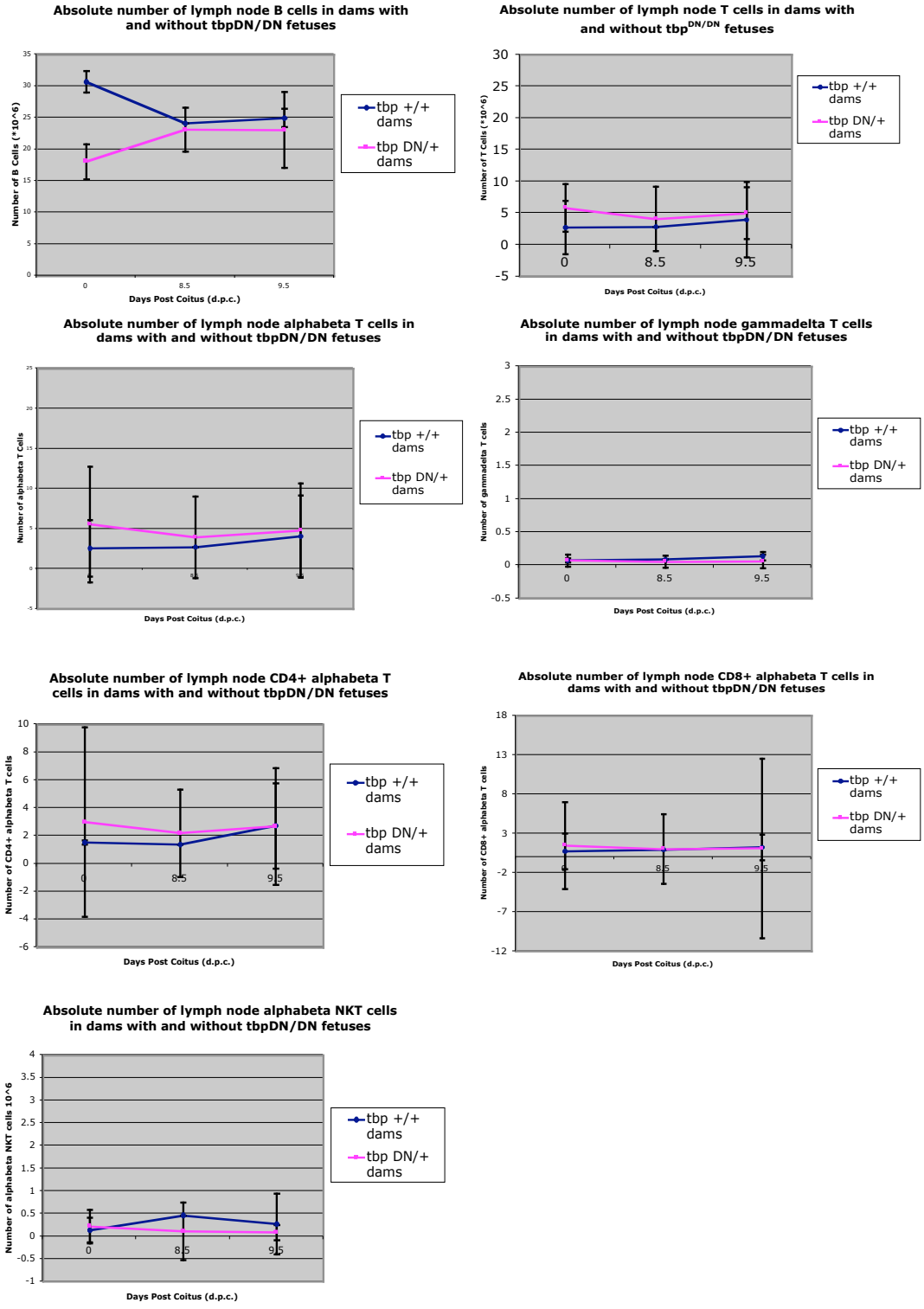


Figure 15. Para-aortic lymph node analysis of adaptive immune cells during early labyrinth development (E8.5-E10.5) in dams with and with *tbp*$^{\Delta N/\Delta N/}$ fetuses.

innate immune system trigger the response while adaptive immune cells modulate the innate factors.

FACS analysis of maternal lymph nodes and immunohistochemical anaylsis of placental sections allowed us to explore these three possible scenarios. Lymphocyte populations in the maternal para-aortic lymph nodes did not change prior to, during, or following rejection (E8.5 to E13.5) in immune wildtype dams carrying *tbp*$^{\Delta N/\Delta N}$ fetuses when compared to dams carrying only *tbp*$^{+/+}$ fetuses (Fig. 15). Immunohistochemical experiments on E8.5, 9.5, and 10.5 *tbp*$^{\Delta N/\Delta N}$ and *tbp*$^{+/+}$ placentas revealed that adaptive immune cells affected in *rag1*-deficient and SCID mutant mouse lines (i. e. B cells and CD8^{+} T cells) are not present in the labyrinth zone of *tbp*$^{\Delta N/\Delta N}$ placentas (data not shown), and these immune cells have the same distribution in other placental regions in *tbp*$^{\Delta N/\Delta N}$ placentas as in *tbp*$^{+/+}$ littermate placentas (data not shown). The maternal B and T cells do not, most likely, recognize and respond to *tbp*$^{\Delta N/\Delta N}$ fetal/placental antigens, and, consequently, do not direct antibody or cytolytic responses toward mutant trophoblast cells. Hence, it can be concluded that the innate immune system is probably initiating the *tbp*$^{\Delta N/\Delta N}$ midgestational abortion with adaptive immune cells maintaining the innate response.

As previously discussed, innate immune responses can lead to fetal rejection. If trophoblast controlled-suppression pathways are not funcitoning (i. e. blocked IDO activity), innate immune mechanisms, such as complement-mediated lysis, will attack trophoblast cells as they invade the uterine wall (E5.5-

7.5) and are first exposed to the maternal blood supply (Caucheteux, et al., 2003; Mellor, et al., 2001; Munn, et al., 1998; Xu, et al., 2000). *tbp*$^{\Delta N/\Delta N}$ fetuses, on the other hand, die in immune wildtype mothers at midgestation in association with a placental labyrinth defect. The labyrinth vasculature does not properly form, and loss of the labyrinth region and placental tissue integrity is seen at the time of fetal death. However, in *rag1*$^{-/-}$ dams, mutant fetuses survive with a fully developed placenta that has a malformed vascular bed suggesting that the continuation of labyrinth misdevelopment may be blocked by the innate immune system in immunocompetent mothers.

CHAPTER 5

SUMMARY AND CONCLUSIONS

To study the function of the vertebrate-specific TBP N terminus, our laboratory created a mouse line carrying a truncated version of the basal transcription machinery. This mutation, *tbp*$^{\Delta N}$, caused a defect in the placentas of *tbp*$^{\Delta N/\Delta N}$ fetuses that led to fetal death at or around midgestation. Previous experiments showed that the *tbp*$^{\Delta N/\Delta N}$ fetal rejection could be blocked if the maternal adaptive immune system was severely compromised, the fetal β_2M-dependent processes were lost, or the *tbp*$^{\Delta N/\Delta N}$ fetuses were given *tbp*$^{+/+}$ placentas. It was also found that the abortive process did not initiate a memory response as would accompany a classical MHC class I/antigen response and that increased survivability of tbp$^{\Delta N/\Delta N}$ mice was not a heritable genetic trait.

Recent data established that tbp$^{\Delta N}$-associated, placental defects arise during the formation of the highly vascularized labyrinth region. In immunocompetent dams, tbp$^{\Delta N/\Delta N}$ placentas are likely targeted by maternal immune factors as the labyrinth begins to abnormally develop between E8.5 and E9.5. In *rag1*-null dams, tbp$^{\Delta N/\Delta N}$ placentas are not targeted for degradation, because the maternal immune system appears to be incapable of responding to the labyrinth defect. Thus, the tbp$^{\Delta N/\Delta N}$ labyrinths in the immunocompromised mothers are allowed to continue development even though the reduced labyrinth vascular network will likely result in fetal growth retardation.

Further analysis of the adaptive immune system did not lead to the determination of an adaptive immune cells initiating *tbp* homozygous mutant rejection. Rather every cell type tested showed either decreased or no effect on *tbp*$^{\Delta N/\Delta N}$ midgestational survivability. Also, immunological analysis of mutant and wildtype placentas and para-aortic lymph nodes in mothers carrying tbp$^{\Delta N/\Delta N}$ fetuses revealed that B cells and T cells were not present in the mutant labyrinth prior to or during midgestational crisis and lymphocyte populations within the lymph node did not expand prior to, during, or following fetal rejection. Since adaptive immune cells do not change in response to rejection, a memory response is not elicited, and tbp$^{\Delta N/\Delta N}$ fetuses still survive in severely adaptive immunocompromised dams, the conclusion was reached that the innate immune system is most likely initiating *tbp*$^{\Delta N/\Delta N}$ midgestational rejection with the adaptive immune system playing a secondary role in the process.

The vertebrate-specifc TBP N terminus has a eutherian-specific function in mice and probably all placental mammals. It is required for proper labyrinth vascularization, since loss of N-terminal activity leads to a malformed labyrinth with a substantially reduced vascular bed. Vertebrate evolution, though, pre-dates placental development, so what role that the N terminus plays in vertebrates remains unknown. However, it can be surmised from our findings that the TBP N terminus may be regulating genes involved in vascular genesis or tissue remodeling of vertebrate-specific tissues and organs.

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