MID- AND LATE-GESTATION LETHALITY IN MICE LACKING THE N TERMINUS OF TATA-BINDING PROTEIN

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ABSTRACT

TATA-binding protein (TBP) is a transcription factor comprised of a 180 amino acid core that is shared by all eukaryotes. TBP also has an N-terminal region that, in vertebrates, is highly conserved. We have generated mice bearing a mutant *tbp* allele, $tbp^{\Delta N}$, that lacks 111 of the 135 amino acids of the vertebrate-specific N terminus. Most homozygous mutants, $tbp^{\Delta N/\Delta N}$, die at midgestation from an apparent defect in their placentas. $tbp^{\Delta N/\Delta N}$ fetuses were rescued at this midgestational crisis if supplied with a wild-type tetraploid placenta. $tbp^{\Delta N/\Delta N}$ fetuses also survived in immune-normal mothers when fetal/placental $\beta_2 m$ expression was genetically disrupted. When reared in immunocompromised mothers, $tbp^{\Delta N/\Delta N}$ fetuses also survived midgestation. These results suggest the N terminus of TBP functions in β_2M-dependent processes and within the placenta to favor immunotolerance during pregnancy at midgestion. Beyond midgestation, $tbp^{\Delta N/\Delta N}$ fetuses that survive in immunocompromised mothers were found to be runted at the perinatal period and died shortly after birth. These latter results suggest that the N terminus of TBP also functions in non-immune processes required for normal birth weight and successful pregnancy.

CHAPTER 1

INTRODUCTION

About TBP

The Function of TBP

Transcription is a process that is strictly regulated in all cells. Initiation of transcription is a multistep process involving coordinated interactions of multiple proteins. In eukaryotes, transcription is carried out by RNA polymerases I, II and III. RNA polymerase (pol) I is localized to the nucleolus and transcribes rRNA, pol II transcribes mRNA from protein-coding genes, and pol III transcribes tRNA genes, the 5S rRNA genes and other small RNAs.

The activities of pol II have generated enormous interest and have been intensely studied. Both *in vivo* and *in vitro* transcription initiation of most protein-encoding genes requires RNA polymerase II, TATA-binding protein (TBP), and a number of general transcription factors including TFIIA, B, D, E, F, and H (Berk, 2000; Chen and Hampsey, 2002; Matsui et al., 1980; Orphanides et al., 1996; Samuels et al., 1982). Transcription initiation of pol II genes begins with the formation of the preinitiation complex at the promoter where binding of the general transcription factor TFIID is stimulated by TFIIA. TFIID then recruits TFIIB to form the DB complex. The unphosphorylated form of RNA pol II, associated with TFIIF joins in to generate the DBpol F complex. Other general transcription factors, TFIIE, TFIIH, and TFIIJ then join to yield the preinitiation complex. The process by which the preinitiation complex forms is dependent on TBP (Berk, 2000). TBP is a part of the TFIID complex and is the subunit that

binds TFIID to the promoter region of a gene by binding the minor groove of DNA. TBP forms a bent saddle-shaped structure (Nikolov et al., 1996) when it binds and opens the minor groove of DNA, creating a 100^{o} turn in the DNA axis. The DNA is then uncoiled within the TATA sequence for transcription to ensue.

In addition to playing a central role in pol II transcription, TBP is required in most multimeric complexes of pol I and III as well. In all these complexes, TBP functions in promoter recognition. Four TBP-containing complexes have been reported to be required for transcription initiation in mammals: SL1, TFIIIB, TFIID, and SNAPc. As discussed above, pol II requires the TBP-containing complex TFIID (Tanese and Tjian, 1993; Weis and D., 1992). Transcription by pol I requires the complex SL1 (Huet and Sentenac, 1992) while pol III requires the TFIIIB complex for initiation of transcription (Dynlacht et al., 1991). Pol II requires the complex TFIID. Together with TFIID, RNA pol II transcribes from both TATA-containing and TATA-less promoters (Hernandez, 1993). Finally, in mammals, the fourth TBP-containing complex, SNAPc, is used by both Pol II and Pol III in the transcription of small nuclear RNAs (snRNAs) (Bosma et al., 1983). Thus, TBP appears to have a nearly universal function in transcription, however, one exception has been demonstrated in *Drosophila* where a TBP-related factor (TRF) is responsible for pol III transcription without TBP (Takada et al., 2000).

With the exception of Eubacteria, all organisms possess TBP. However, across species, differences in genomic and gene expression complexity exist. Unlike in eukaryotes, where TBP functions at the core of different large multiprotein complexes to orchestrate transcription from three different RNA pols (Hernandez, 1993), TBP in Archaea appears to function as a single-subunit entity that plays a role with a single RNA polymerase to transcribe all genes (Qureshi et al., 1997). Thus, TBP, through evolution has likely established new and diverse

interactions with proteins associated with the basal transcription machinery to orchestrate the transcription of increasing numbers of genes.

Evolution of TBP

In Archaea, TBP appears to function as a single-subunit entity that plays a role with a single RNA polymerase to transcribe all genes (Qureshi et al., 1997). In eukaryotes, however, TBP has come to reside at the core of several large, complex gene expression machines to orchestrate the transcription of large numbers of genes (Hernandez, 1993). It is therefore likely that TBP, through evolution, has had to be able to interact with multiple more proteins associated with the basal transcription machinery in eukaryotes.

Recently, studies to more closely evaluate the phylogenetic distribution of the TBP N-terminus have placed the appearance of this domain at or near the origins of the first vertebrates (Bondareva and Schmidt, 2003). This puts the appearance of the conserved vertebrate N terminus coincident with many other vertebrate characteristics including vertebrae, neural crest, nasal ducts and gill arches (Delarbre et al., 2002; Janvier, 2004; Neidert et al., 2001). Although many of the genes that participate in the formation of these characters have been elucidated, many of them precede the existence of those characters. For example, while some transcription factor families that participate in the development of neural crest cells have been identified, these gene families function in pathways that are more ancient than the neural crest cells themselves (reviewed in Meulemans and Bronner-Fraser, 2004). Other examples of genes that have been co-opted to take on new functions include AP-2γ, Dlx3, and Hand1 (Cross et al., 2003a).

Thus, as the functions of the TBP N-terminus are elucidated in

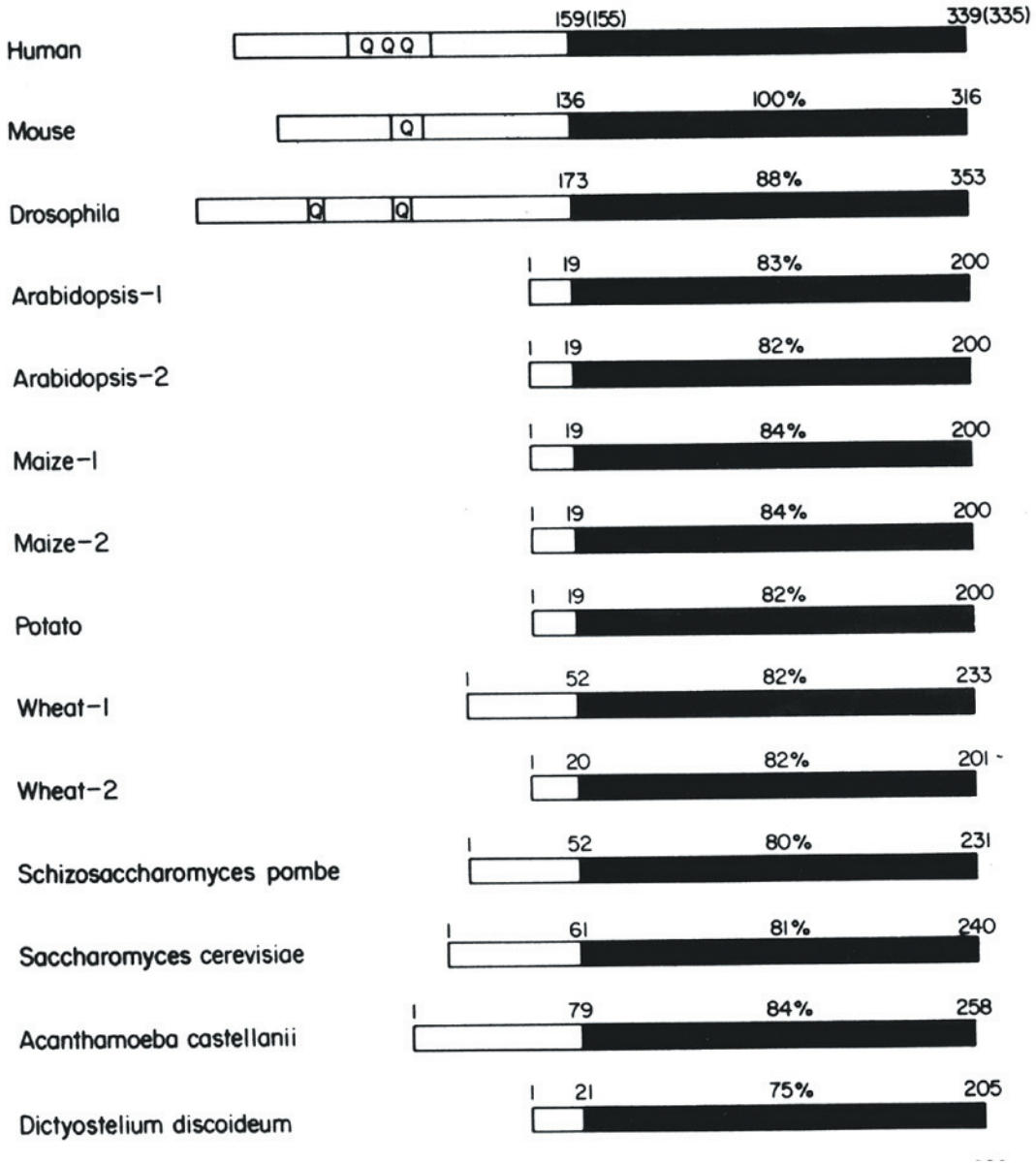

Figure 1. Schematic representation of C-terminal and N-terminal domains of TBP from multiple species. The C terminus is represented by the black box and percentages are the identity shared with human. The N terminus is represented by the white box. Glutamine is represented by Q. Modified from Hernandez, 1993.

vertebrates, it will become important to distinguish between those pathways that have co-opted the N terminus recently in evolution from more ancient pathways, where redundant systems for the N terminus may exist.

The C terminus

The carboxy- (C-) terminal domain of TBP is 180 amino acids in length and forms the core region that binds DNA. This core performs most of the known functions of TBP and is conserved across eukaryotes and Archaea. Sequence comparisons of the C-terminus reveal a high level of conservation among all eukaryotes (Hernandez, 1993) (Fig. 1). Further, crystal X-ray analyses of this portion of the protein reveals a bent saddle-shaped structure (Nikolov et al., 1996) that is very similar in all eukaryotic species (Hernandez, 1993). This portion of the protein is indispensable for life. In developmental studies that employed gene targeting to remove all or part of the *tbp* gene, mouse embryos that lack TBP do not develop past the blastocyst stage due to inhibition of pol I and III transcription (Martianov et al., 2002). Thus, the level of amino acid conservation, along with these gene knock-out studies, suggest that the function of the TBP C terminus is essential for eukaryotic life.

The N terminus

Although eukaryotic species possess various N-terminal domains of the TBP protein, a conserved version exists exclusively in vertebrates (Bondareva and Schmidt, 2003; Tamura, 1991). TBP sequences from different species can be analyzed through NCBI-GenBank. This database shows that the sequence of the N terminus is nearly identical in all vertebrates. Between mouse and human,

the amino acid sequence identity of the N-terminal domain is nearly 100% (Fig. 1). In contrast, non-vertebrate metazoans, including protostomes and lower deuterostomes, do not possess an N terminus that shares sequence identity with the vertebrate-specific N terminus (Bondareva and Schmidt, 2003). Unlike the C terminus, the crystal structure of the N terminus has yet to be determined (Zhao and Herr, 2002), presumably because this domain is a flexible structure when not bound to its protein interacters.

The mouse TBP N terminus is encoded almost entirely by exon 3 of the *tbp* gene (Nakashima et al., 1995; Ohbayashi et al., 1996). A comprehensive examination of the N terminus resulted in a breakdown of this region into four subdomains. They include a central polyglutamine repeat, the two regions that flank the glutamine repeat, and a junction region adjacent to the conserved C terminus of the protein, which is an imperfect repeat region of the sequence (PXT)n, where X is generally M, A, or I. Among metazoan taxa, the PXT repeat varies in length as does the length of the glutamine repeat region. The junctional region is thought to serve as a connector that integrates phyla-specific properties of the N terminus with the universal functions known to be performed by the C terminal portion of TBP (Bondareva and Schmidt, 2003).

Although the function of this domain has yet to be fully elucidated, some studies have reported functions for this portion of the protein. Many of these studies have been performed using human TBP. One such study has provided evidence that the N terminus is required in the assembly of the small nuclear RNA (snRNA) activating complex (SNAPc). SNAPc is required for transcription of U6, a snRNA gene involved in splicing processes. Interestingly, snRNA promoters are also highly conserved in vertebrates (Hernandez, 1992). Studies have also shown that the N terminus can inhibit TBP binding to DNA (Mittal and

Hernandez, 1997). In another study, the high mobility group protein, HMG-1, has been shown to bind the glutamine (Q) repeat within the N terminus of human TBP. This interaction was demonstrated to increase the affinity of TBP for the TATA element by approximately 20-fold (Das and Scovell, 2001). Kinetic studies of this region indicate that it facilitates DNA-binding of the C-terminus by inducing conformational changes (Zhao and Herr, 2002) and in chicken DT40 cells, heterozygous disruption of TBP appears to implicate the N terminus in cellular growth rate regulation (Um et al., 2001).

Functions of N termini in Other Species

Other taxa possess TBP N-terminal domains that are unrelated to that in vertebrates. Yeast has an N terminus that is unrelated to the conserved vertebrate counterpart. Investigations involving N-terminal deletion in yeast resulted in enhanced TBP-TATA-box association and TBP dimerization. Additionally, these studies demonstrated that the yeast N terminus acts positively in combination with a specific region of the TBP core domain that interacts with other protein(s) (Lee and Struhl, 2001). Studies in *Drosophila* have demonstrated possible roles for the N terminus in RNA pol II-mediated transcription. Here it was shown that the N terminus strongly activated transcription when fused to a GAL4 DNA binding domain (DBD), but these properties were inhibited in the full-length protein (Um and Manley, 2000). Whether N-terminal functions from such studies can be used to better understand the vertebrate-specific N terminus is uncertain, given the high degree of variability in this domain in eukaryotes.

Other *tbp* Gene Family Members

Three other *tbp* gene family members, termed TBP-related factors, or TRFs, have recently been discovered (Davidson, 2003; Hochheimer and Tjian, 2003). Two of these proteins have been shown to have distinct functions from that of TBP. TRF1, the first TRF to be identified, has only been found in neuronal and germ cells of *Drosophila* (Crowley et al., 1993). TRF1 is a transcription factor involved in RNA polymerase II transcription and can bind TFIIA and TFIIB *in vitro*. In a complex with BRF, a core component of the RNA pol III transcription factor TFIIIB in *Drosophila*, TRF1 has an essential function in the transcription of tRNA, 5S, and U6 RNA genes, entirely without TBP (Takada et al., 2000).

Another TRF, which has been designated TRF2 (also known as TLF, TLP, or TRP), appears to be broadly distributed in metazoans including *Drosophila*, the nematode *Caenorhabditis elegans*, frog (*Xenopus laevis*), mouse (*Mus musculus*), and humans (Maldonado, 1999; Moore et al., 1999; Ohbayashi et al., 1999; Rabenstein et al., 1999; Teichmann et al., 1999). Loss-of-function studies have demonstrated a requirement of TRF2 in some taxa. In *Danio rerio, Caenorhabditis elegans*, and *Xenopus laevis*, TRF2 can replace TBP at certain somatic cell loci (Davidson, 2003). TRF2 is an essential transcription factor required for transcription of a restricted subset of genes in the early *C. elegans* embryo (Dantonel et al., 1999) and development past the mid-blastula stage in *Xenopus* embryos requires the presence of this protein as well (Veenstra et al., 2000). In *Mus musculus*, TRF2-deficiency leads to male sterility because of a severe defect in spermiogenesis (Martianov et al., 2001; Zhang et al., 2001). Like TBP, both TRF1 and TRF2 interact with general transcription factors such as TFIIA and TFIIB (Rabenstein et al., 1999). The DNA binding domain of TRF2

has diverged from that of TBP, however, and does not interact with the TATA box (Rabenstein et al., 1999). For example, pairs of phenylalanines in TBP found in the TATA-box binding region that bend DNA upon binding are not conserved in either TRF1 or TRF2. Thus, it is unlikely that TRF1 and TRF2 have redundant roles with TBP. Rather, they likely function with pol II to transcribe classes of genes with promoter elements distinct from those with a TATA-box.

Finally, TRF3 is the most recent TBP-like family member discovered. The C terminus of TRF3 is nearly identical to TBP, including residues required for DNA binding and interaction with general transcription factors. Also like TBP, the N-terminal region of TRF3 is divergent and the gene is present in vertebrates from fish to humans. Although functions for this protein have not yet been reported, the presence and high conservation of TRF3 in vertebrates suggests that, like TBP, TRF3 plays an important vertebrate-specific role. Whether TRF3 shares redundant roles with TBP has yet to be elucidated (Persengiev, 2003). However, the existence of such TBP family members may help to explain some previous findings that have suggested TBP-independent mechanisms for RNA polymerase II transcription (Davidson, 2003) and should be considered in the interpretation of all TBP-deletion studies.

The Immune System

Evolution of the Immune System

All organisms are subject to pathogens and parasites from their environment. While some immune functions are unique to specific taxa, many have been found to be phylogenetically related and some are similar

to those found in mammals. For example, the discovery of a GATA factor in *Drosophila* involved in hematopoiesis in vertebrates implies that some aspects of the molecular mechanisms underlying hematopoiesis are shared between deuterostomes and protostomes (Rehorn et al., 1996). In vertebrates, macrophage cells are large leukocytes that function in phagocytosis, antigen processing and presentation, secretion of cytokines, and antibody-dependent cell-mediated cytotoxicity (reviewed in Gregory and Devitt, 2004). Circulating macrophages have also been shown to be involved in innate immunity of both mammals and some invertebrates (Du Pasquier and Flajnik, 1999). Additionally, conservation of macrophage receptors can be found in different organisms (Pearson, 1996). For example, a macrophage receptor for apoptotic cells exists in *Drosophila,* that mediates removal of apoptotic cells by macrophages (Franc et al., 1996).

The adaptive immune system arose in an ancestor to all living vertebrates (Bernstein et al., 1996). In jawed-vertebrates, or gnathostomes, multiple strategies have been employed to recognize and protect against foreign invaders. Defense mechanisms include those that can be inhibited by self but activated in the absence of self. Also, a repertoire of antigen-specific receptors, depleted of antiself activity, and secreted immunoglobulin functions to provide immunity (reviewed in Viret C, 1999). Together, these components in jawed-vertebrates are referred to as the adaptive (or aquired) immune system.

The genes that define adaptive immunity include T cell receptors (TCR), immunoglobulins (Ig), Major Histocompatibility Class (MHC) I/II, and recombination-activating genes (RAG) and are present in the oldest jawed vertebrates (Laird et al., 2000). In contrast, no homologous genes, molecules, or mechanisms have been identified in jawless vertebrates (or agnathans), such as

hagfish and lamprey, or in invertebrates. Furthermore, hagfish and lamprey lack the lymphoid organ, thymus (Flajnik and Kasahara, 2001) where the T cells of jawed vertebrates undergo selection and education.

However, the lack of identical immune components in these organisms does not necessarily negate the existence of some other form of an adaptive immune system. A verstion of saaptive immune responses have been reported in lamprey and hagfish (Forey and Janvier, 1993). In a search for lymphocyte-specific genes in lamprey, ESTs for transcription factors, cell-surface molecules, and other proteins that are expressed in lymphocytes of jawed-vertebrates were identified (Uinuk-ool et al., 2002). Recently, a novel system of highly variable lymphocyte receptors was identified that may serve a role in the recognition of pathogens (Pancer et al., 2004). This system could represent an analogous (but not homologous) agnathan version of the jawed-vertebrate adaptive immune system.

Innate and Adaptive Immune Systems

The Innate Immune System

All metazoan organisms have evolved complex immune defense systems to repel invasive microbes that can parasitize or kill them. Innate immunity is an evolutionarily ancient defense mechanism (Fearon and Locksley, 1996) and is the most rapidly acting. Only in vertebrates has adaptive immunity evolved: the alternative system for pathogen recognition and elimination (reviewed in Beutler, 2004) involving antigen-recognition receptors and immunological memory (discussed in detail below).

The innate immune system provides the first line of defense in the immune response, functioning to attack foreign antigens. It is apart of all normal

individuals at all times and does not increase with repeated pathogen exposure. This system rapidly mounts an attack using invariant receptors to repel a pathogen or to hold it in check until the adaptive immune system can come in. Innate immunity in vertebrates is largely dependent on professional immunocytes that engulf and destroy pathogens. These include mononuclear phagocytes and polymorphonuclear phagocytes. The mononuclear phagocytes are the macrophages and dendritic cells, derived from blood monocytes, which are highly efficient at presenting antigens to T cells of the adaptive immune system. The polymorphonuclear phagocytes include neutrophils, basophils, eosinophils and are important in the containment of infection. Neutrophils are specialized killers that possess a broad array of weapons with which to destroy their microbial prey. Eosinophils and basophils are more immediately concerned with the production of mediators that shape the inflammatory milieu, and are responsive to cytokines enhanced by the adaptive immune system (reviewed in Beutler, 2004).

Other responses of the innate immune system include the alternative pathway of complement activation (antibody independent), cytokines (reviewed in Magor and Magor, 2001) and antimicrobial peptides like defensins and cathelicidins (Ganz and Lehrer, 1998; Lehrer and Ganz, 2002a; Lehrer and Ganz, 2002b). Finally, natural killer, or NK, cells also function early in the host response to infection without the need for prior immunization or activation. These cells function, in part, with the ability to recognize the presence or absence of self MHC molecules on the surfaces of cells. This recognition determines the actions of NK cells.

Although the innate immune system is often referred to as “non-specific”, several lines of evidence suggest this name is misleading. On the contrary, the innate immune system has evolved several strategies of self/nonself

discrimination deciphered by receptors that either induce or inhibit an immune response (Medzhitov and Janeway, 2002). Examples of such receptors include expression of the glycosylphosphatidylinositol (GPI)-anchored protein and CD14 on the surface of monocytes, macrophages, and polymorphonuclear leukocytes that specifically bind LPS (Hailman et al., 1994). Many more examples of receptor specificity are provided by studies on Toll receptors – a large family of receptors used for signaling in defense mechanisms (reviewed in Magor and Magor, 2001). Different pathogen molecules lead to the activation of distinct Toll receptors, resulting in a unique immune response. Within the Toll receptor family, specific activation occurs through recognition of LPS of Gram-negative bacteria (Poltorak et al., 1998), of Gram-positive bacteria and yeast (Underhill et al., 1999), of bacterial flagellin (Hayashi et al., 2001), of CpG DNA (Hemmi et al., 2000), and of the peptidoglycan component of bacterial cell walls (Ozinsky et al., 2000).

Adaptive Immunity

Adaptive immune responses are different from those of the innate immune system in that they are mediated by clonal selection of antigen-specific lymphocytes and result in immunological memory. Adaptive immunity functions only after several days of an infection because time is required for lymphocytes to differentiate and proliferate in the presence of an antigen.

The two major types of immune cells in adaptive immunity are T cells and B cells (also known as lymphocytes). Lymphocytes display an enormous variety of cell surface receptors to recognize and respond to an unlimited number of pathogens, a feature that is considered the hallmark of the “adaptive’’ immune system (Market and Papavasiliou, 2003). B cells are lymphocytes derived from hematopoietic stem cells of the bone marrow (Carsetti, 2000; Chung et al.,

2001). They express cell-surface immunoglobulin molecules as receptors for antigen, but also secrete immunoglobulin as soluble antibody to provide defense against infection in extracellular spaces. B cells become activated when they encounter an antigen that expresses epitopes specifically recognized by its cell surface receptor, Ig (reviewed in Carsetti et al., 2004). T cells are another class of lymphocytes and undergo differentiation in the thymus and recognize their specific antigen when associated with MHC molecules on the surface of antigen presenting cells (APCs) (Margulies, 1999). These lymphocytes become activated upon interaction of T cell receptor (TCR)/CD3 molecule complexes with its ligand presented in the groove of MHC molecules. T cells also express cell-surface receptors such as CD4 and CD8, which have been used to classify T cells into different subpopulations. While the hallmarks of these activated lymphocytes are diverse antigen (Ag)-receptor repertoires, T and B cells could not possess such diversity without the somatic gene rearrangement of their V(D)J genes performed by two very important enzymes: RAG-1 and RAG-2. The action of these enzymes on V(D)J rearrangements are indispensable for function of the mature lymphocyte (reveiwed inGellert, 2002).

Interdependence of the Two Arms of Immunity

Although the responses of innate immunity can act without priming or the cooperation of other immune components, the full breadth of an immunological response involves both innate and adaptive immunity. The interdependence of the innate and adaptive immune system can be demonstrated when removal of one part results in a lack of activity by the other aspect of immune. For example, animals deficient for mature T and B cells exhibit decreased NK cell activation (Carnaud et al., 1999). Early in the infection process, phagocytes release cytokines and other inflammatory mediators such as tumor necrosis

factor-α (TNF-α), to recruit more phagocytic cells and other leukocytes. These secreted molecules can also function to activate NK cells and initiate lymphocyte development and migration to sites of infection or tissue damage. Toll-like receptors (TLRs), a component of the innate immune system, can mediate control of adaptive immunity (Iwasaki and Medzhitov, 2004). Cytokines produced early in infection influence differentiation and participation of certain subsets of T cells. Activated T cells can regulate the growth and effector functions of other T cell subtypes, activate macrophages at the site of an infection and interact with B cells. Naïve B cells can then become activated and begin secreting antibodies in an antigen-specific manner. As a result of mounting an adaptive immune response, a state of immunological memory is established, due to a clonally expanded population of antigen-specific T and B lymphocytes. Thus, a series of interconnected events orchestrated by both the innate and adaptive arms of immunity result in host defense and lasting immunity. Some selected components of the immune system are discussed in detail below.

Selected Immune System Components

Recombination Activation Gene-1 (RAG-1)

V(D)J recombination, which joins variable (V), diversity (D), and joining (J) gene segments (Tonegawa, 1983), is the mechanism by which the genes for antigen receptors in lymphocytes are assembled. V(D)J recombination occurs at seven different loci: the immunoglobulin (Ig) heavy chain, κ and λ light chains in B lymphocytes (Tonegawa, 1983), and in TCR α, β, γ, and δ chains in T lymphocytes (Davis and Byorkman, 1988).

The *rag-1* gene was identified as an agent of V(D)J recombination in NIH 3T3 cells (Shatz and Baltimore, 1988; Shatz et al., 1989) and in later studies

the structurally unrelated gene, *RAG-2*, was identified (Oettinger et al., 1990). Both RAG-1 and RAG-2 proteins initiate V(D)J recombination (Oettinger et al., 1990; Shatz et al., 1989) (Fig. 2). The RAG proteins initiate recombination by introducing a nick precisely between the Recombination signal sequences and the coding segment. This generates a free 3′ OH, which is used to attack the opposite strand in a transesterification reaction that forms a hairpin (coding end) and a signal end terminating in a flush double-strand break. The resulting broken DNA ends (blunt signal ends and hairpin coding ends) are then joined to form the rearranged products by the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs) (discussed further below) (Brandt and Roth, 2002).

These proteins act in concert to cleave DNA at the junctions between antigen receptor coding segments and the conserved recombination signals that specify sites of recombination (McBlane et al., 1995; van Gent et al., 1995). RAG-1 mediated DNA cleavage proceeds in two steps: first, one DNA strand is nicked between the recombination signal sequence (RSS) heptamer and the coding sequence, and second, a transesterification reaction resulting in a free hydroxyl group at the 3’ end of the coding sequence attacking a phosphodiester on the opposite strand (van Gent et al., 1996a; van Gent et al., 1996b). The result of these reactions leaves two DNA ends: a signal end, terminating in a blunt, 5’-phosphorylated, double-strand break, and a coding end, terminating in a hairpin (McBlane et al., 1995).

Mice null for RAG-1 have been generated with the use of targeted homologous recombination in embryonic stem cells (Mombaerts et al., 1992). Mice homozygous for this mutation (*rag*$^{-/-}$) are fertile and have proven healthy and indistinguishable from their normal littermates. However, they have small lymphoid organs that do not contain mature B and T lymphocytes, although NK

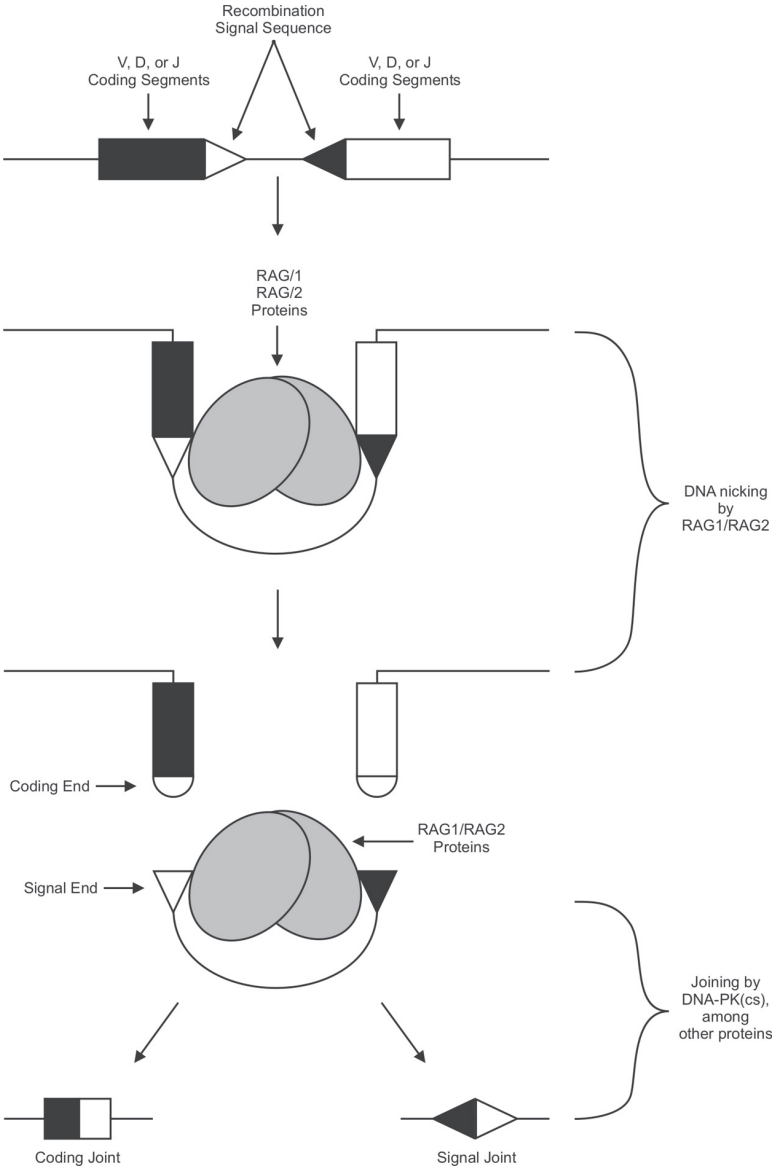

Figure 2. Overview of V(D)J recombination. V, D, or J coding segments (boxes on the DNA strand) are selected for recombination because they are located adjacent to Recombination Signal Sequences (triangles), which are bound by RAG1 and RAG2 proteins (ovals). The RAG proteins bring the two recombination signals together to form a synaptic complex prior to cleavage. DNA cleavage creates hairpin coding ends and blunt signal ends; after cleavage, it is thought that the RAG proteins remain associated with signal ends and coding ends in a post-cleavage complex, although the coding ends may not be as tightly bound. Joining factors including DNA-PKcs are involved in the formation of coding and signal joints. Both the RAG-1 and scid mutations generate defects in this pathway that lead to a lack of mature T and B cells in mice. Adapted from Brandt and Roth (2002).

cell levels are normal (Mombaerts et al., 1992). Although *RAG-1* transcripts have been reported in the brain of mice (Chun et al., 1991), no overt structural or behavioral abnormalities have been reported for *rag*$^{-/-}$ mice (Mombaerts et al., 1992). A similar phenotype is described in RAG-2-deficient mice (Shinkai et al., 1992).

RAG-1 vs. SCID

Like RAG-1 deficient mice, severe combined immunodeficiency (SCID) mice, lack mature T and B cells (Bosma et al., 1983; Bosma and Carroll, 1991). Also like the RAG-1 deficient mice, SCID mice do possess normal levels of NK cells (Hackett et al., 1986). SCID mice lack T and B cells due to a mutation that disrupts the same pathway that mutations in *RAG-1* and *RAG-2* disrupt: double-stranded break rejoining during V(D)J recombination (Blunt et al., 1995) (Fig. 2). The *scid* mutation arose from a single T to A transversion at Tyr-4046 (Araki et al., 1997; Blunt et al., 1996) in the gene for a catalytic subunit of DNA-dependent protein kinase (DNA-PKcs). DNA-PKcs is encoded by the XRCC7 gene, is a member of the phosphoinositol (PI) kinase family and has been shown to play an important role in repair of DNA damage incurred by γ-radiation and other double-stranded break inducing agents. DNA-PKcs is recruited at the site of DNA lesions through interactions with the regulatory complex Ku70/80 that is responsible for binding to the ends of DNA (Gottlieb and Jackson, 1993). As a result, mice with this mutation possess a generalized defect in double-strand break repair as well as being severely impaired in carrying out V(D)J recombination for lymphocyte development (Blunt et al., 1995).

B_2M

The molecule, beta-2-microglobulin (β_2M), is a 99 amino acid polypeptide

and is the necessary small subunit for conformational stability and cell surface expression of most classical and non-classical MHC I molecules (Margulies, 1999; Pamer and Cresswell, 1998). The MHC is a set of tightly linked genes found on chromosome 6 in humans and chromosome 17 in mice (Margulies, 1999). β_2M is encoded by a separate gene on chromosome 2. Studies on intracellular transport of class I molecules suggest that the heavy chains associate with β_2M (the light chain) immediately after their synthesis in the rough endoplasmic reticulum (Sege et al., 1981). This association is required for presentation of antigenic peptides to T cells (Townsend and Bodmer, 1989). Additionally, β_2M is required for expression of products from the nonclassical *Qa/Tla* genes that share sequence homology with the classical class I antigens (Koller and Smithies, 1989). Thus, β_2M plays an essential role in the presentation of many types of antigen to maternal T cells.

In adult mice, almost all cells in the body express β_2M and MHC I molecules (Pamer and Cresswell, 1998). Both β_2*M* and *MHC I* genes are highly expressed in lymphoid tissues, and at relatively lower levels in other tissues such as brain and muscle (Chamberlain et al., 1988). There are many studies that have elucidated the pattern of both β_2M and MHC I in the developing fetus and placenta as well (Jaffe et al., 1990; Jaffe et al., 1991; Philpott et al., 1988). Regulation and/or absence of MHC gene expression during early development of the mouse, particularly in subsets of extraembryonic cell lineages that contribute to the placenta, is thought to be critical in maternal tolerance of the fetal allograft.

However, it can be concluded that MHC I molecules are not required for successful pregnancy. Mice lacking β2M ($\beta_2 m^{-/-}$) have been generated by disrupting exon 2 of the β*2m* gene (Koller and Smithies, 1989). Although these mice fail to present MHC I on their cell surfaces regardless of which MHC I large

subunit genes are expressed (Dang and Heyborne, 2001; Jaffe et al., 1992), β_2M-null mice are fertile and have successful pregnancies with no embryonic abnormalities (Koller and Smithies, 1989).

MHC I

The MHC is also referred to as the HLA complex in humans and the H-2 complex in mice (Margulies, 1999). Classical MHC I molecules in mice (MHC Ia) are cell-surface glycoproteins consisting of a 40-45 kDa heavy chain and include the H2-K, H2-L, and H2-D proteins that represent classical transplantation antigens. MHC Ia and are expressed in nearly all somatic cells of adult mouse and human and are highly polymorphic (Stroynowski, 1990). The heavy chain consists of three extracellular domains designated α1, α2, and α3, a transmembrane domain, and a cytoplasmic tail that is 30-40 amino acid residues in length (Flavell et al., 1986). The classical MHC I genes are polymorphic, meaning that this locus has a high frequency of genetic variants (Klein, 1986). It is thought that this polymorphism in MHC genes may exist as a selective advantage by having a pool of antigen-presenting molecules that could allow for the binding and presentation of a broad array of antigenic peptides .

MHC I molecules present endogenously derived peptides. Antigen presentation takes place when cytosolic proteins are first degraded in the multiproteolytic proteosome complex (Margulies, 1999) to generate peptide fragments. These fragments are then delivered to the endoplasmic reticulum by the transporter associated with antigen processing (TAP) where they assemble into MHC I/β_2M complexes. From here, they pass through the Golgi to the cell surface for presentation to T cells (Margulies, 1999). Studies of biosynthesis and intracellular transport of class I molecules indicate that the heavy chains associate with β_2M proteins after their synthesis in the rough

endoplasmic reticulum (Sege et al., 1981). This association leads to substantial conformational changes of the heavy chain that plays a role in its efficient transport to the cell surface. Without β_2M, MHC I is not expressed on the cell surface, but instead remains intracellular and eventually gets degraded (Sege et al., 1981; Williams et al., 1989).

Once on the cell surface, MHC I molecules play an essential role in presentation of viral and tumor-associated antigens to cytotoxic T lymphocytes (CTLs) (Doherty et al., 1984), $CD8^+$ T cells that form conjugates with and kill target cells (reviewed in Waterhouse et al., 2004) and thus are indispensable in adaptive immune responses. T cells that recognize MHC I/antigen complexes generally have $\alpha\beta$ chains. Recognition by T cells occurs through the coreceptor for MHC I molecules, CD8, which plays a role in both the activation of mature T cells and thymic development of MHC I-restricted lymphocytes (Zamoyska, 1994). Depending on the endogenous antigen that an MHC I molecule is presenting, a T cell that engages these molecules will either determine that a cell is “self” or “non-self”. Expression of these molecules on cell surfaces also influences NK cell activity. In mice, NK cells recognize MHC I molecules on potential target cells via receptors of the Ly49 family (Karlhofer et al., 1992; Mason et al., 1995; Stoneman et al., 1995). Thus, MHC I molecules are important in innate responses of the immune system as well.

Because cell surface expression of MHC I requires the accompaniment of β_2M, it is generally thought that these two genes are expressed in the same tissues. However, as discussed below, there are some exceptions to colocalization of these two molecules in the developing fetus and placenta.

In addition to the classical MHC genes and are nonclassical MHC (MHC Ib) genes that are also on mouse Chromosome 17. Nonclassical MHC

Ib, which far outnumber the Ia genes in mice, are expressed in a much more tissue restricted manner and are relatively non-polymorphic (Stroynowski, 1990). Examples of nonclassical MHC I molecules include CD1 (Porcelli and Modlin, 1995) and H2-M3 (Lindahl et al., 1997). H2-M3 presents relatively short peptide antigens selectively binds and presents peptide antigens that possess an N-terminal formyl moiety. CD1 molecules have a limited tissue-specific expression pattern, present lipid and glycolipid antigens (reviewed in Brigl and Brenner, 2004; Porcelli and Modlin, 1999), and are recognized by both $\alpha\beta$ and $\gamma\delta$ T cells.

Beyond the nonclassical MHC genes are the minor histocompatibility genes. These genes are not limited to just one chromosome like the major MHC genes but are found throughout the genome of both mice and humans (Gubarev et al., 1996; Zuberi et al., 1998). A well-studied example of minor histocompatibility genes in mice is the H3 complex, on Chromosome 2. Like the major MHC genes, this locus is important for understanding mechanisms underlying non-self antigen recognition in transplantation rejection between MHC-matched mouse strains (Zuberi et al., 1998).

The Placenta

Evolution of the Placenta

Placentae occur widely in Vertebrata as an essential part of reproduction and development (Blackburn, 1998; Mossman, 1987) (Fig. 3). Across taxa, the placenta functions for similar purposes: to establish and maintain gas and nutrient exchange between the fetus and mother or external environment. The mammalian chorioallantoic placenta, the result of fusion between the chorion

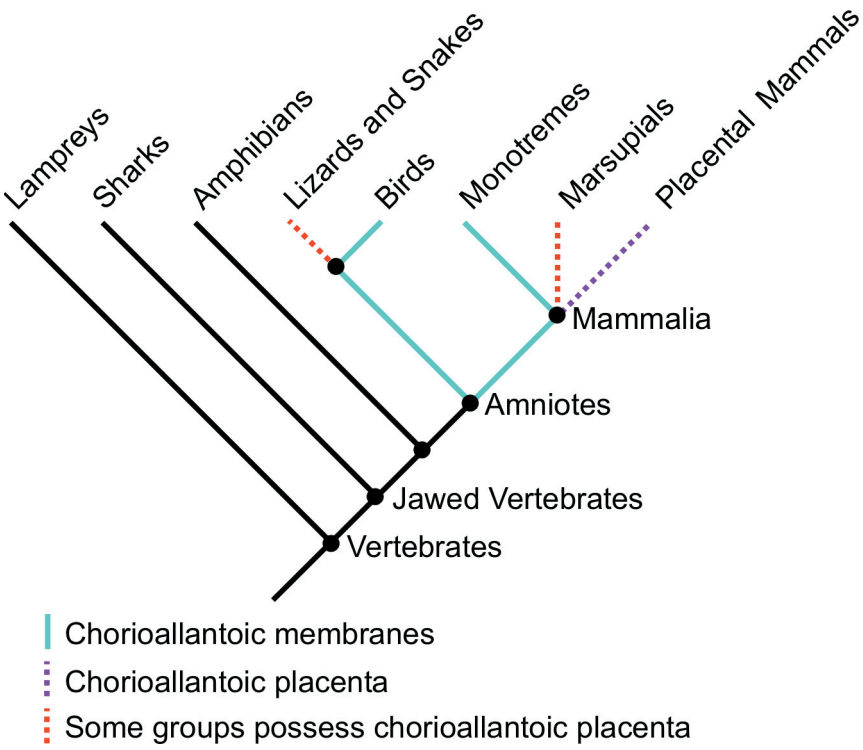

Figure 3. Phylogenetic distribution of chorioallantoic placentae in vertebrates.

and allantois, evolved from the extraembryonic membranes found in reptiles (including birds) where a chorioallantoic membrane exists under the eggshell and exchanges gas and calcium from the shell to the developing fetus (Blackburn, 1993). Vertebrate placentae are thought to have evolved multiple independent times (Blackburn, 2000; Stewart and Thompson, 2000). For example, placentae are known to occur in over 28 genera of sharks (Dulvy and Reynolds, 1997) and in reptiles, placentation is thought to have originated on over 100 separate occasions (Blackburn, 1992). Even in mammals, comparative anatomy of mammalian placentae reveal diverse forms and suggest that this organ evolved independently (Kaufmann and Burton, 1994).

The mouse placenta is derived from multiple cell lineages and is a composite of both fetal and maternal tissues. Trophoblast cells of the placenta

in mammals, derived from the trophoblast lineage, invade into the uterus to establish an intimate contact between fetus and mother (Cross et al., 2003a). To regulate the maternal response to such invasion, additional roles have evolved in the mammalian placenta, including the production of hormones to alter systemic maternal functions and growth factors to alter the local uterine environment. Many of these functions are observed across different vertebrate taxa and are thought to have existed since very early vertebrate evolution (Painter et al., 2002) (Callard and Koob, 1993; Hughes, 1993; Renfree, 1993). Gene knockout and transgenic studies have greatly increased our knowledge of novel genes and gene families important in these processes, shedding light on development, function, and molecular components of mammalian placenta.

Development and Function of the Mouse Placenta

Formation of the Mouse Blastocyst

Twelve hours after fertilization on E0.5 (it is assumed that fertilization occurs at the mid-point of the dark cycle, usually midnight, for mice kept on a 12h light-12h dark light cycle), a sequence of cellular divisions create a ball of undifferentiated cells termed the morula. By E3.5, the blastocyst forms at the 32-64 cell stage. Two cell types can be distinguished at this point: the inner cell mass (ICM) and trophectoderm. The ICM consists of the innermost cells of the blastocyst. They are undifferentiated stem cells that will eventually give rise to the entire embryo proper as well as the mesenchymal (mesodermal) components of the placenta such as stromal cells and blood vessels. The inner cell mass differentiates into the three germ layers of the embryo during gastrulation: endoderm (or hypoblast), mesoderm, and ectoderm (or epiblast). Primitive endoderm begins to differentiate at approximately E4.0 from the free

surface of the ICM of the blastocyst. This lineage has been demonstrated to only differentiate into the extraembryonic parietal and visceral endoderm of the yolk sac. Primitive endoderm does not contribute to the endodermal tissues of the fetus (Gardner, 1982). At approximately E4.5, the core ICM cells of the blastocyst derive the primitive ectoderm that divides rapidly and generates all tissues of the fetus proper (Theiler, 1972; Theiler, 1983).

Cells on the outside of the blastocyst differentiate into an epithelium called trophectoderm which are the first cells of the trophoblast lineage that will contribute exclusively to the epithelial portion of the developing placenta. Those trophectoderm cells that are adjacent to the ICM will become polar trophoblast and cells of the trophectoderm not adjacent to the ICM will become mural trophoblast cells. At implantation, the mural trophectoderm stop dividing, become large and, in mice, can increase their ploidy up to 1000N. These cells form the primary tophoblast giant cells. Polar trophectoderm remain diploid and continue to proliferate. These cells can later become polyploid giant cells and chorion cells and will generate a considerable amount of the placenta (Hogan et al., 1994; Theiler, 1972; Theiler, 1983).

Implantation

Implantation initates when embryos at the blastocyst stage breach the uterine epithelial cell layer, creating the beginning of intimate interactions between the zygote and maternal systems. At this time, the pregnant uterus undergoes a radical structural transformation. Decidualization, the proliferation and differentiation of uterine stromal cells, increases the thickness of the uterine wall by approximately five-fold. Endometrial stromal cells, at the site of implantation begin to differentiate into primary decidual cells. Cells that are more distal to the implantation site differentiate into secondary decidual cells

(Theiler, 1972; Theiler, 1983). This differentiation and rearrangement of cells allows for the placenta to be positioned in the mesometrial decidua (Hunt et al., 1997). Uterine NK (uNK) cells traffic to the site of implantation, rapidly proliferate and become localized to the mesometrial side of each implantation site. Here uNK cells form the lymphocyte-rich structure known as the metrial gland (or mesometrial lymphoid aggregate of pregnancy) (Adamson et al., 2002; Ashkar et al., 2000). Starting at E9.5, major decidual arteries begin to undergo gestation-induced modification characterized by thinning of the arteries, an increase in lumen diameter, and vessel elongation (Forsburg and Dowell, 1992). On the embryo side, it is at this point that formation of the mature placenta, heart and vascular systems must quickly transpire (Cross, 2001). Until this can occur, the visceral yolk sac plays an important nutritive role in early developmental stages (Jaffe et al., 1990).

The mature placenta

The mature placenta is established in mouse by day E10.5. The zygote-derived components of the mature placenta consists of trophoblasts (extraembryonic ectoderm) and endothelial and stromal cells (extraembryonic mesoderm)(Rodriguez et al., 2004). Within the placenta, the trophoblast lineage has differentiated and reorganized to generate cell subtypes with distinct endocrine, vascular, immune, and transport function capabilities (Cross, 2001). Like humans, the mouse placenta is hemochorial; maternal blood is in direct contact with placental trophoblasts (Wooding and Flint, 1994). Further development of the placenta relies on the interdependence of allantoic mesodermal cells and chorion trophoblast cells (Hatano et al., 2003).

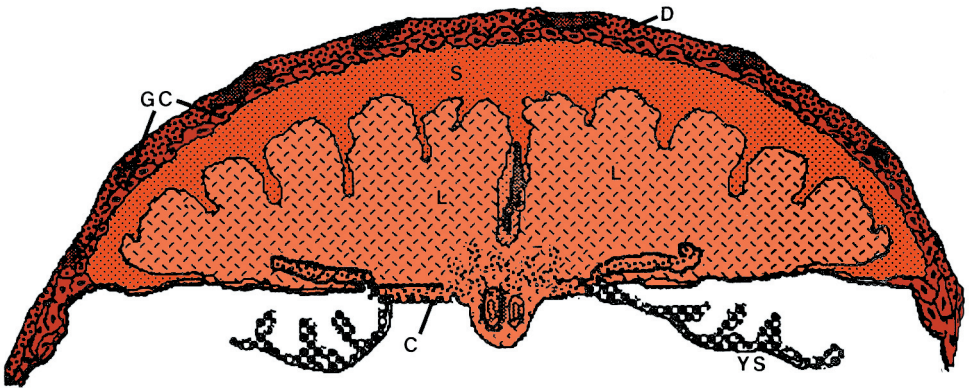

Figure 4. The mouse placenta. D = maternal decidua, GC = giant cells, S = spongiotrophoblast, L = labyrinth, C = chorionic plate, YS = yolk sac. Modified from Zuckermann, et al., 1986.

placenta and gene expression

Once fully developed, the placenta proper can be divided into four layers: the giant cell layer, the spongiotrophoblast layer (also called the junctional or spongy zone), the labyrinth and the chorionic plate (Fig. 4). The trophoblast giant cells form the outermost layer of the placenta, are directly adjacent to maternal tissues, and perform several functions. They are a very invasive cell and are the first to mediate implantation and the process of migration into the uterine wall (Cross, 2001). A striking feature of trophoblast giant cells is that they are extensively polyploid (up to 1000N) due to endoreduplication, the process by which rounds of DNA replication ensue in the absence of mitosis (MacAuley et al., 1998). Giant cells are therefore easily recognizable in placental sections, due to their extremely large nuclei. Giant cells not only secret immunosuppressive agents during implantation, but also produce angiogenic factors such as VEGF and Proliferin that are thought to target maternal vasculature and promote ingrowth of new vessels to the implantation site (Adamson et al., 2002). Giant cells also produce *Hand1* and *Mash2*. The transcription factor, *Hand1*, is

essential for differentiation and also regulates the promoter for the giant cell-specific hormone, placental lactogen I gene (*Pll*). *Hand1* has been shown to induce trophoblast growth arrest and giant cell transformation (Cross et al., 1995). In contrast, *Mash2* has the opposite effect of *Hand1* by maintaining giant cell precursors in an undifferentiated state. *Mash2*-deficient concepti have more giant cells than their wild type controls (Guillemot et al., 1994). Other genes expressed by giant trophoblast cells include *Ets2*, which play a role in the implantation process by blastocysts (Yamamoto et al., 1998), and *Gata2/3*, that are required for fetal development and regulate placental lactogen-1 and proliferin (Tsai et al., 1994). Giant cells produce four members of the prolactin (PRL)/growth hormone (GH) family in the mouse. They are placental lactogen I (PL-I), placental lactogen II (PL-II), proliferin (PLF), and proliferin-related protein (PRP) (Ogren and Talamantes, 1988). Proliferin is an angiogenic factor that, when disrupted, results in reduction of maternal blood flow to the implantation site (Ma et al., 1997). Finally, co-expression of cyclins E1 and E2 are essential for giant cells to undergo endoreplication. Double knock-outs of *CCNE1* and *CCNE2*, the genes encoding E1 and E2, respectively, result in embryonic lethality at mid-gestation (Parisi et al., 2003). Double homozygous mutants can be rescued with tetraploid complementation, suggesting that the lethal defect is placental.

The spongiotrophoblast layer was once thought to simply be a structural zone to support the underlying villi (vascular processes), however, it has been shown to secret a number of polypeptide hormones (Linzer and Fisher, 1999; Soares et al., 1996). The spongiotrophoblast layer is a compact cellular zone that is perfused only by maternal blood and which, together with the giant cells, apposes maternal decidua (Zuckermann and Head, 1986). By E13.5

of development, the spongiotrophoblast layer becomes that which is directly adjacent to the maternal component of the placenta as the giant cells by this point are no longer maintained (Jaffe et al., 1990).

Labyrinth morphogenesis begins with interposition of allantoic cells and fetal blood vessels into the chorionic plate. The labyrinth contains both trophoblasts and mesodermally derived cells that are embryonic in origin. Labyrinth development can be separated into four distinct steps: chorioallantoic fusion (attachment of the allantois to the chorionic plate) which beings at E8.5, early morphogenesis and syncytiotrophoblast differentiation, expansion of the labyrinth, and vascularization of the labyrinth (Cross, 2001). The labyrinth begins to function as a nutrient transport unit around E10.5. It is here that gas, nutrient, and waste exchange occurs between the mother and fetus. Thus, abnormal development or function of the labyrinth can result in impaired fetal development (Rodriguez et al., 2004). There are several genes that, through the use of knockout mice, have been reported to be critical for later steps in growth of the labyrinth. These mutations result in a reduced labyrinth size often accompanied by abnormal histological findings and include *Dlx3*, *Esx1*, *Fgfr2*, *JunB*, *Lifr*, *Ppar*γ, *Mek1*, *Hsp90*β, *Rxr-*α, *Hgf*, *c-Met*, *Vcam1*, and *Wnt2* (Cross, 2000; Cross et al., 1994; Rinkenberger et al., 1997), Additionally, formation of the labyrinth is controlled by a distinct genetic pathway governed by the transcription factor *Gcm1*, a homologue of the *Drosophila glial cells missing* gene. *Gcm1* encodes a novel transcription factor that is expressed in the labyrinth layer at the time of chorioallantoic fusion and continues to be expressed through gestation until E17.5 (Basyuk et al., 1999). Mutation of *Gcm1* results in the inability of chorionic trophoblast cells to fuse to form syncytiotrophoblast. As a result, embryonic lethality occurs at E10.5 due to the absence of the labyrinth layer of the placenta

(Anson-Cartwright et al., 2000). Additionally, although chorioallantoic fusion occurs normally in *Gcm1* mutants, the chorionic plate remains flat, resulting in the absence of villi formation. Since expression of *Gcm1* in chorionic trophoblast cells depends on contact with allantoic mesoderm, this mechanism explains why chorionic villous development does not progress in the absence of mesodermal cells. Blood vessels develop in *Gcm1* mutants, but its branching into a network is restricted because of the block to chorionic morphogenesis.

The region closest to the fetus is called the chorionic plate. It is here that the yolk sac inserts and the umbilical cord originates. Fetal capillaries derived from the umbilical blood vessels branch from the chorionic plate and intermingle with a network of sinuses containing maternal blood in the labyrinth (Zuckermann and Head, 1986).

Finally, although the vascular system of the placenta is not a distinct layer, it is one of the most critical features of the placenta. Thus, a thorough description of placental function would be incomplete without it. Blood vessel formation in the placenta occurs by angiogenesis and is regulated by multiple genes. In knock-out studies, mutations in the *Arnt* and *Vhl* genes demonstrate the importance of these genes in vascular defects of the placenta. Additionally, *Esx1* and *JunB* mutations produce vascular defects specifically in the labyrinth (Gnarra et al., 1997; Kovats et al., 1990; Li and Behringer, 1998; Schorpp-Kistner et al., 1999). Several genes that are involved in development and remodeling of placental vasculature are regulated by IFN-γ, commonly produced from resident uNK cells. These include inducible nitric oxide synthase (iNOS), endothelial (e)NOS, and endothelin-1, all of which are major regulators of vascular contractility (Boehm et al., 1997).

The Maternal Components of the Placenta

The placenta is a composite organ that is composed of zygote-derived cells that are in close association with maternal tissues. The maternal components associated with the placenta include the decidua basalis on the uterus side of the placenta, the decidua capsularis on the embryo side of the placenta, and the metrial triangle (also known as the mesometrial triangle or metrial gland) (Kruse et al., 1999b), a region rich in uterine natural killer (uNK) cells (also called granulated metrial gland cells) (Croy and Kiso, 1993).

During early stage pregnancy (around E9.5), uNK/granular lymphocyte-related leukocytes (or granulated metrial gland (GMG) cells), maternal monocytes/macrophages (Matthews et al., 1985), neutrophils and granulocytes are common residents of the uterus (Adamson et al., 2002). B and T cells are relatively rare early in pregnancy, but proportions of T cells increases with gestational age (Kearns and Lala, 1985). In the pregnant uterus, the most abundant maternal lymphocyte population is uNK cells (Croy and Kiso, 1993; King and Loke, 1991; Kruse et al., 1999b) (Fig. 5) and can comprise up to 20% of the maternally-derived mesometrial triangle (established by E10.5) (Croy and Kiso, 1993). uNK cells have a large granular morphology and are found at the implantation site. Several studies have demonstrated the importance of uNKs for successful pregnancy by assaying mice lacking NK cells altogether. Tgε26 mice (NK- and T-cell deficient) and $Rag2^{-/-}\gamma_c^{-/-}$ mice (deficient in NK, T, and B cells) fail to generate metrial glands (Croy et al., 2000) and the decidual arteries in these mice do not undergo remodeling, resulting in failed pregnancy (Ashkar et al., 2000; Guimond et al., 1997). However, reconstitution of Tgε26 mice with the bone marrow of T and B cell-deficient SCID mice rescues these defects during

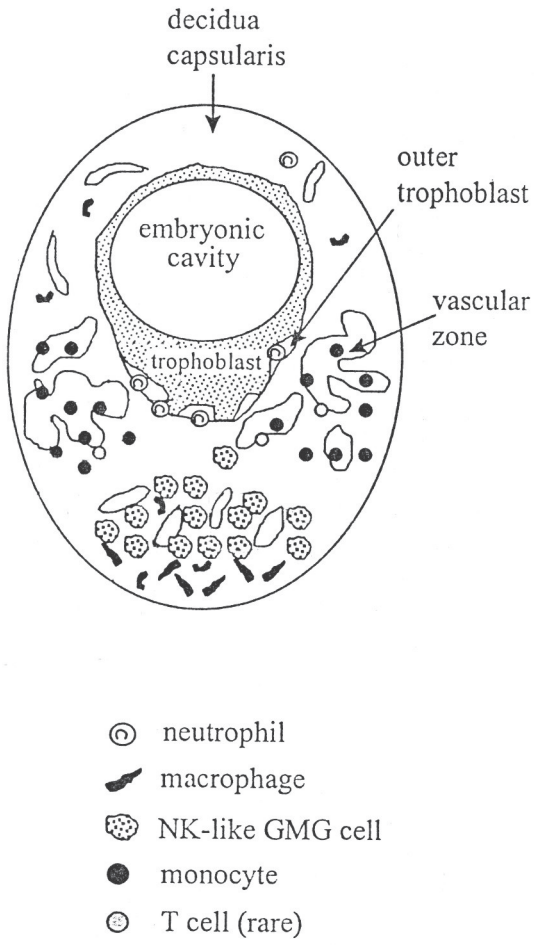

Figure 5. Maternal cells of the pregnant decidua. Modified from Kruse, et. al., 1999b.

pregnancy, implicating the uNK cell population as the primary cause of pathology (Guimond et al., 1998). uNK cells are a major source of interferon (IFN)-γ (Platt and Hunt, 1998; Saito et al., 1993) which plays roles in pregnancy-induced decidual artery remodeling (Ashkar et al., 2000). IFN-γ has also been shown to regulate α_2-macroglobulin, a regulator of proteases and cytokines produced from the mesometrial deciduas that are important for successful pregnancies. Thus, the uNK cell component from the maternal system, along with the IFN-γ produced from these cells is essential for modification of decidual arteries required for

successful pregnancy (Ashkar et al., 2000).

An expanded subpopulation of NKT cells has also been reported to be in the peri-implantation uterus. These cells express the NK marker NK1.1 and are Vα14^{+} and CD4^{-}/CD8^{-}. In mice, these cells are generally restricted by the non-classical MHC molecule CD1d, express TCRα chains with invariant usage of Vα14 and Jα281 genes, and recognize a variety of non-mammalian glycolipids including α-galactosylceramide (α-GalCer) (Matsuda and Kronenber, 2001). Vα14 NKT cells are suspected to react with an unidentified fetal antigen that appears to regulate their expansion (Dang and Heyborne, 2001). Neutophils are almost exclusively limited to the leading edge of enzymatic digestion created by invading trophoblast. Neutrophils at this site are likely involved in phagocytosis of cellular debris from decidual cells killed by invading trophoblast (Parr et al., 1990).

Another source of maternal cells within the placenta is the maternal vasculature. The placenta is a highly vascularized organ and is rich in both fetal and maternal blood (reviewed in Cross et al., 2003b). By E10.5, maternal blood vessels pass through the giant cell and spongiotrophoblast layers and reach the labyrinth zone where they form close contact with fetal capillaries. Large surface areas between maternal vessels and fetal capillaries allow for gas and nutrient exchange in the labyrinth (Kruse et al., 1999a).

The Placenta Evades a Maternal Immune Response

The close association of fetal and maternal tissues puts the maternal immune system in direct contact with foreign fetal antigens. However, the placenta is not considered a “classical” privileged site because the mother becomes sensitized to paternal antigens during pregnancy (van Kempen et al.,

2001) as evidenced by alloantigen-specific alterations in maternal T and B cell phenotypes (Jiang and Vacchio, 1998; Tafuri et al., 1995). Numerous studies have demonstrated that upon recognition of fetal antigen, immunoreactive T and B cells are deleted or down-modulated during pregnancy (Ait-Azzouzene et al., 1998; Tafuri et al., 1995; Zhou and Mellor, 1998). Because of these observations, it is not likely that the maternal immune system "tolerates" a pregnancy merely due to either a lack of fetal antigen presentation or from maternal immunological "ignorance" of the pregnancy. Regulation of the maternal innate immune system in response to pregnancy is also critical for fetal survival (Hunt et al., 2000; Sacks et al., 1999). Indeed, it appears that, instead, the maternal immune system is involved in intimate interactions with the fetal/placental unit.

It has often been hypothesized that the nature and constituents of the maternal immune response during pregnancy is modulated by the fetus (Dang and Heyborne, 2001). For example, in humans, fetal extravillous cytotrophoblasts (called trophoblast giant cells in mice) at the maternal-fetal interface lack classical MHC class Ia molecules, due to TAP deficiency to avoid recognition by maternal alloreactive T cells (Faulk and Temple, 1976; Rodriguez et al., 1997). Without TAP, cytosolic peptides are not transported to the endoplasmic reticulum where they can be cooperatively folded into an MHC I molecule and eventually presented on the cell surface (Heemels and Ploegh, 1995). Instead these trophoblasts in human express a MHC class Ib molecule HLA-G, which is thought to provide protection from maternal uterine NK cell – mediated lysis (Kovats et al., 1990); (Lanier, 1999).

Studies that have focused on the expression of these MHC class Ib molecules at the maternal-fetal interface have increased our knowledge about how fetal tissues may regulate maternal immune responses. In mice, the cloning

of a new nonclassical MHC class Ib gene, termed blastocyst MHC, has been described that is expressed in both blastocyst and placenta (Sipes et al., 1996). This gene resembles human HLA-G in structure which, unlike classical MHC I molecules, is expressed on trophoblast cells at the fetal-maternal interface (Kovats et al. 1991) and may present paternal antigen to the maternal immune system. Some have hypothesized that such nonclassical MHC I molecules may play a role in immune surveillance to allow the maternal immune system to recognize virally infected trophoblast cells (Wei and Orr 1990). Others have hypothesized a role for these molecules in protecting trophoblast from NK cell-mediated lysis by allowing trophoblast cells to be recognized as self (Kovats et al. 1991) Nevertheless, it is now well known that the close association of maternal and fetal/placental tissues exposes the maternal immune system to paternally-derived antigens and many studies are focusing on how fetal/placental antigen tolerance occurs in pregnancy (Erlebacher, 2001; Loke and King, 2000). The mechanisms by which this occurs, however, are not yet clear.

Immunosuppressive Agents of the Placenta

Another mechanism that the placenta apparently uses to evade a maternal immune response is the expression of immunosuppressive agents. Ablating the expression of these immunosuppressive agents has been shown to result in spontaneous abortion in mice (Xu et al., 2000; Yamamoto et al., 1998). For example, specific trophoblast cell types have been shown to produce progesterone, metal proteases, and inhibitors of complement (Cross et al., 1994; Munn et al., 1998; Van Vlasselaer and Vandeputte, 1984; Xu et al., 2000). The complement inhibitor, Crry, is a member of a family of ubiquitously expressed molecules that negatively regulates complement components C3 and C4 (Molina et al., 2002). Disruption of *Crry* lead to deposition of complement associated

with placental inflammation, leading to embryonic lethality. *Crry*$^{-/-}$ embryos can be rescued by rearing them in mothers that are deficient for C3 (*C3*$^{-/-}$), which lack the ability to mount a normal complement reaction (Xu et al., 2000). In humans, trophoblasts have been shown to express complement regulatory proteins, pressumably to circumvent complement-mediated lysis as well (Holmes and Simpson, 1992). Expression of indoleamine 2,3-dioxygenase (IDO), a tryptophan-catabolizing enzyme, has also been demonstrated in trophoblast cells (Munn et al., 1998). IDO is thought to reduce local concentrations of tryptophan below the threshold required for normal T cell function (Munn and Armstrong, 1993; Munn et al., 1996) at the fetal-maternal interface (Kamimura et al., 1991). In the absence of tryptophan, cell cycle progression is halted at a mid-G1 arrest point in T cells (Munn et al., 1999). Requirements for IDO have been demonstrated for fetal survival in studies using 1-methyl-tryptophan (Munn et al., 1998). This drug, when administered to pregnant mice, results in abortion of allogeneic but not syngeneic concepti. The effects of 1-methyl-tryptophan only occur when T cells specific for allogeneic paternal H-2 antigens are present and the drug is also associated with complement deposition at the maternal-fetal interface. The investigators of these studies suggest that 1-methyl-tryptophan causes allogeneic abortions by inhibiting the effects on IDO.

The immunosuppressive agents prostaglandin E_2, TGF-β, and IL-10 are secreted locally at the fetal-maternal interface. It is thought that secretion of these agents function to protect against attack from maternal lymphocytes (Clark et al., 1990; Wegmann et al., 1993). Other studies have demonstrated that in humans, trophoblast cells express L-selectin, a carbohydrate binding protein, which mediates interactions with the uterus that may be critical in establishing a successful pregnancy (Genbacev et al., 2003). Immune evasion by the fetus is

also demonstrated in studies that demonstrate expression of FasL at the fetal-maternal interface which is thought to act by preventing passage of activated $FasR^+$ immune cells (Hunt et al., 1997). FasL is known to restrict migration of activated lympyhocytes into other immune-privledged regions such as testis and the anterior chamber of the eye by delivering a death signal to lymphocytes through their Fas recceptors (Suda et al., 1995). In *gld* mice, a mutant line that lacks functional FasL, extensive leukocytic infiltrates and necrosis at the decidual-placental interface are observed from E10.5, eventually resulting in increased resorption and small litter sizes (Hunt et al., 1997). Together, these studies demonstrate close links, at the molecular level, between processes important to reproductive and immune functions.

Expression of $\beta_2 m$ in Mouse Placenta and Embryo

The fetus and its placenta represent a foreign entity to the mother's immune system. In classical tissue graft rejection, the host immune system recognizes foreign antigen via MHC I presentation. Because the fetus has been historically compared with classical tissue graft rejection, *MHC I* and $\beta_2 M$ expression have been extensively studied through pregnancy. *In situ* analysis of gene expression reveals that β_2M mRNA expression first appears in extra-embryonic derivatives of the early primitive streak stage embryo and is restricted to the ectoplacental cone and chorion (Jaffe et al., 1991). $\beta_2 M$ expression continues in these tissues through the formation of the placenta. In the visceral yolk sac at E8.5, hemopoietic precursors are present and by E10.5 in this tissue, expression is detected in all cell types including endoderm, extra-embryonic mesoderm, and hemopoietic cells within capillary networks (Jaffe et al., 1990). Throughout development, $\beta_2 M$ mRNA is detected in the maternal decidua basalis

and in all cell layers of the placenta, with the exception of the trophoblast giant cells (Jaffe et al., 1990). By E13.5, the spongiotrophoblast layer comes in direct contact with the maternal decidua as the giant cell layer disappears (Faria et al., 1991; Jaffe et al., 1990). Spongiotrophoblast cells, from this point on, remain the most strongly β_2M expressing layer of the mature placenta (Jaffe et al., 1990).

In the fetus proper, β_2M mRNA is first detected at E9.5 in all somatic tissues and expression is highest in the fetal liver (Jaffe et al., 1990), the site of hematopoiesis. Production of β_2M in the fetus continues to increase in all tissues through development, but liver remains the strongest expressing organ.

Expression of *MHC I* in Mouse Placenta and Embryo

While there is evidence that the maternal immune system recognizes paternal antigen during pregnancy (Jiang and Vacchio, 1998; Tafuri et al., 1995; van Kempen et al., 2001), some tissues of the mouse placenta do not express classical MHC I on the cell surface (Hedley et al., 1988; Jaffe et al., 1991). This absence is thought to play a role in tolerance of the paternally-derived antigens in utero. Additionally, expression of *MHC I* does not necessarily correlate temporally or spatially with the expression of β_2M. Studies using in situ hybridization methods have analyzed MHC I expression patterns in the mouse embryo and placenta during development (Jaffe et al., 1991). *MHC I* transcripts are first detected at E9.5 in trophoblast giant cells (Jaffe et al., 1991) (It should be noted here that these studies detected expression of *MHC I* mRNA, not the expression of MHC I protein on the cell surface. Trophoblast giant cells are in fact devoid of classical MHC I protein on the cell surface, which is discussed in detail below). Like β_2M, detection of *MHC I* expression is high in the maternal decidua basalis, throughout development. Giant cells, however, are the only site

in the placenta that does not express β_2*M.* By E12.5-E13.5 high levels of *MHC I* mRNA are detected in spongiotrophoblast and at lower levels in the labyrinth (Jaffe et al., 1991; Philpott et al., 1988).

In the embryo proper, *MHC I* mRNA is faintly detected at E10.5. As is the case for β_2*M*, *MHC I* expression continues to increase through development in the embryo and by E13.5, the fetal liver expresses the highest levels of *MHC I*. High levels of expression of these genes in fetal liver is likely due to the fact that fetal liver is a major site for T and B lymphocyte development (Boersma et al., 1981).

The studies discussed above demonstrate that expression of β_2*M* and *MHC I* transcripts in the developing mouse embryo and placenta are not coincident in all cell types. It has been speculated that the differential expression between *MHC I* and β_2*M* is due to regulation of MHC I via a wide range of cytokines including both IFN and TNFα (David-Watine et al., 1990). Reportedly, there are TNFα responsive elements in the promoter regions of *H-2K*^{b} genes but not in that of β_2M (Israel et al., 1989). Additionally, in rats, it has been demonstrated that many tissues during pregnancy including uterine epithelium, decidual cells, and trophoblast cells produce TNFα (Yelavarthi et al., 1991).

Such promoter differences could account for how MHC I and β_2*M* could be differentially expressed. Yet, it has been proposed that spatially different expression of these two genes may not necessarily prevent MHC I and its antigen from getting to the cell surface. In general it is thought that cell surface expression of MHC I protein without β_2M is not impossible. However, one study reports that H-2K^b heavy chains could be detected on the cellular surfaces of β_2M-defective cell culture lines (Williams et al., 1989), although defects in intracellular transport without β_2*M* were ~70% penetrant. Additionally, those

heavy chains that did reach the cell surface were not folded in their native state. It has also been suggested that MHC I molecules may not require β_2*M* to be transcribed in the same cells in order to associate with it: β_2*M* has been shown to freely exchange at the cell membrane (Bernabeu et al., 1984; Kimura et al., 1983). The authors proposed that proper cell surface expression of MHC I on a cell (like giant cells) could occur while in association with β_2M produced from adjacent cells (like decidua basalis cells). However, such an association has yet to be demonstrated *in vivo*, and is not likely given that heavy chains require the association with β_2M intracellularly after their synthesis in the rough endoplasmic reticulum (Sege et al., 1981)

Statement of the Hypothesis

During evolution, the components of the basal transcriptional machinery have changed dramatically, as new genes and gene families arose to perform an increasing number of functions. In order to control transcription of new genes, novel mechanisms by which regulators could communicate with RNA polymerase likely evolved. For example, additional surfaces on the basal transcription machinery could function as sites for novel protein-protein interactions resulting in the coordination of new gene pathways that function in placenta or other developmental processes.

TBP is known to play a central role in almost all eukaryotic transcription. Unlike the basal transcription machinery, the core TBP protein has changed very little through evolution. In an ancestor to vertebrates, TBP acquired the large N-terminal domain that has remained conserved in vertebrates ever since. Near this same time in evolution, characteristics such as a vertebral column, neural

crest cells and others first appeared (Neidert et al., 2001). It is likely that the N terminus functions in pathways that originated during this point in evolution. Because of its conservation, we have hypothesized that this domain could be a new surface that functions with and has coevolved alongside regulators of vertebrate-specific genes.

In previous work from our group, most of the N-terminal domain of TBP was removed using targeted homologous recombination in mouse embryonic stem cells. Mice homozygous for this mutation have normal basal transcription function and cellular physiology. However, these mice bear a placental defect and most die at midgestation. In Chapter three, we described a placental defect at midgestation and show that homozygote lethality is due to a maternal immune response. Homozygous mutants can be rescued by 1) supplying wild-type tetraploid placentas, 2) by eliminating the maternal adaptive immune response, and 3) disrupting fetal/placental β2M. In chapter four, a second crisis for homozygous mutant mice is described that apparently does not involve a maternal adaptive immune response and results in post-natal lethality. Homozygous mutants survive to term in immunocompromised females, but are runted and die immediately after birth.

The results of these studies, along with the fact that the TBP N terminus is conserved in vertebrates, suggest that this domain functions in a signaling pathway(s) that regulates placental functions required for immunotolerance during pregnancy.

CHAPTER 2

METHODS

The Targeting Construct

The targeting vector to remove most of the N terminus of TBP was constructed using targeted homologous recombination to replace the endogenous mouse *tbp* gene (Fig. 6). The targeting vector was introduced into 129/J mouse embryonic stem (ES) cells using the Cre/*loxP* system (Hobbs et al., 2002). Cre, a bacteriophage P1 recombinase, is able to catalyze recombination between *loxP* DNA sequences (Sternberg and Hamilton, 1981). The vector contained a *loxP*-flanked MC1-neo cassette (Deng et al., 1993) and was inserted upstream of all the known promoters of the *tbp* gene (Schmidt et al., 1997). The MC1-neo cassette served in negative selection for G418 drug-resistant clones. Additionally, two tandem copies of the FLAG epitope tag were included in the vector to replace most of exon 3, which encodes the majority of the N terminus. The resultant mutant allele encoded a truncated protein containing the first 24 amino acids of TBP, followed by two tandem repeats of the FLAG tag, and the entire C-terminus (amino acids 136–316) of TBP. All introns and splicing signals (Schmidt et al., 1997) were left intact, as well as all known transcription regulatory signals (Ohbayashi et al., 1996; Schmidt et al., 1997). ES cell clones that contained the mutant version of exon 3 along with the neo cassette were injected into C57Bl/6 mouse blastocysts to create chimeric mice. Mice that carried the *tbp* mutation in the germline were bred to produce animals heterozygous for the mutation ($tbp^{\Delta N/+}$).

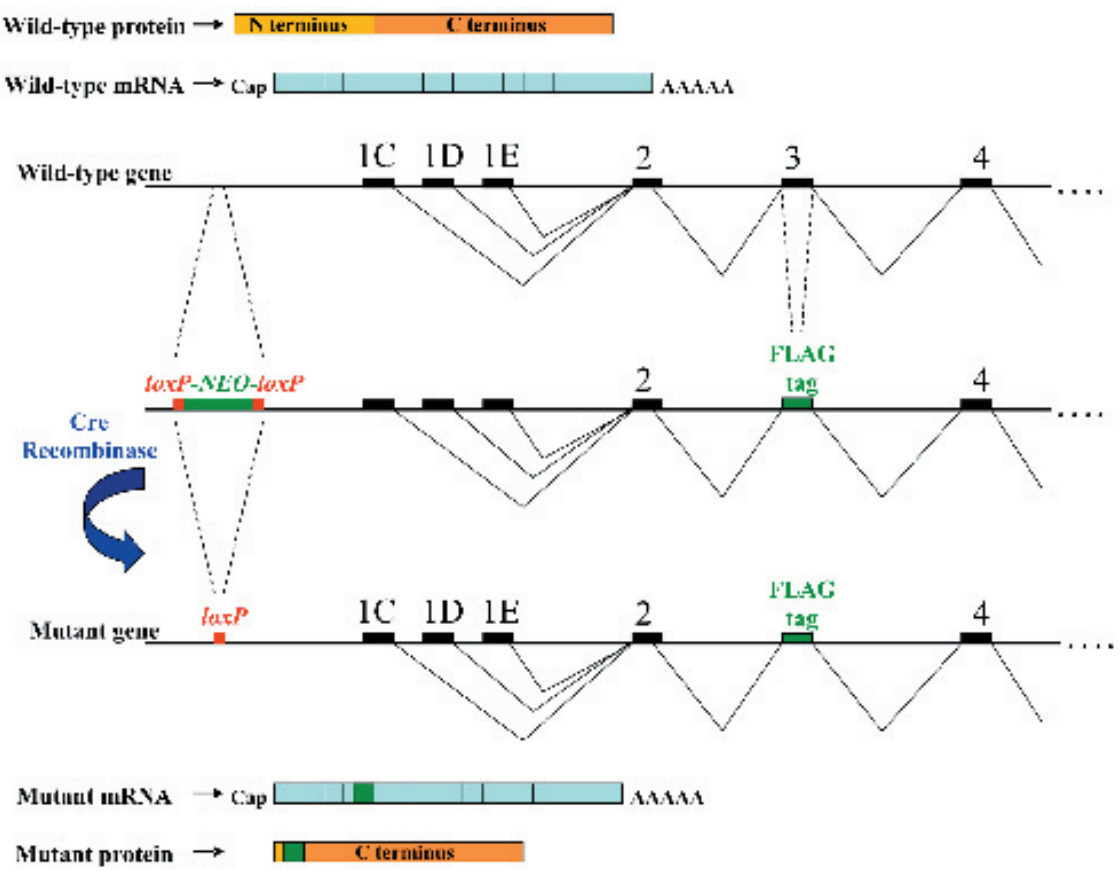

Figure 6. The targeting vector to remove amino acids 25-135 from the N terminus of TBP. Modified from Hobbs, et. al., 2002. The 5′ end of the wild-type *tbp* gene is shown as first black horizontal line at top. Black boxes on line represent exons 1C, 1D, 1E for alternate promoter/first exons, and exons 2, 3, and 4. Below black horizontal lines are the alternate splicing patterns for *tbp*. Bottom black line represents the mutant gene of *tbp* after Cre-*lox*P recombination (blue arrow). Dotted lines indicate which regions of the wild-type allele is replaced by either a *lox*P-flanked *neo* gene (red and green box) or two tandem copies of the FLAG epitope tag (green box). After Cre recombination, one copy of *lox*P is left (red box). Wild-type *tbp* mRNA is shown at top of diagram in light blue and the mutant version of *tbp* mRNA is shown at the bottom in light blue, including the FLAG epitope tag (green box). Wild-type TBP protein is shown at top in yellow (N terminus) and orange (C terminus). At bottom is the mutant version of the TBP protein showing that all but the first 24 amino acids of the N terminus (yellow) have been removed.

Genotyping

To obtain genotypes of mice, molecular analyses were performed on tail snips (obtained at weaning) or fetal tissues. For fetal genotypes, three independent tissue samples were harvested from each fetus (typically tail and two limbs) and were analyzed separately. Samples were digested using 150 ug/ml PK in 50mM Tris HCl, pH 7.5, 100mM NaCl, 100mM EDTA, and 1% SDS. After a 16 hour digestion at 55°C, NaCl was added to 2M and the samples were incubated on ice for 45 minutes. Tubes were then centrifuged for 5 minutes at 14,000 RPM at 4°C in a Labnet Spectrafuge. The supernatant was extracted using a 1 ml pipette and transferred to tubes containing 1ml 100% EtOH. After mixing, floating DNA pellets were transferred into 200ul of 1mM Tris-HCl, pH 7.5, 0.5M EDTA. PCR was used to determine which alleles of *tbp*, *rag-1*, β_2*m* and *8q* (a molecular marker used to follow the fate of tetraploid cells, discussed below) were possessed by each fetus. For genotyping the *tbp* allele, two-primer assays were used and for the *rag1*-, β_2*m*-, and *8q*-alleles, three-primer assays were used. PCR reactions were performed on an Eppendorf Mastercycler. All PCR products were separated on a 1.5% agarose/1X TBE/ethidium bromide gel and DNA bands were visualized on a UV transilluminator and were photographed with a photo-doc system.

PCR for *tbp* Genotypes

Two different sets of primers for the *tbp* gene were used. The first amplifies a region upstream of the *tbp* promoters that, in mutant alleles, contains either the *loxP*-flanked MC1-neo cassette, or the single *loxP* site left after Cre recombination. These primers result in 150 base-pair product from the wild-type

tbp allele and either a 1,350 base-pair (before recombination with Cre) or a 222 base-pair (after *loxP*-neo has been recocombined out) product from the mutant allele. The program used was 94°C for 30 seconds for one cycle; 32 cycles at 94°C for 30 seconds, 61.5°C for 30 seconds, 72°C for 2 minutes and 30 seconds; and one cycle of 72°C for 5 minutes.

The other *tbp* gene primer set amplifies a region across exon 3 of *tbp* where two copies of the FLAG epitope tag replace most of the N terminus in the mutant allele. This second primer set results in a 133 base-pair product from animals that are positive for the *tbp* mutation and a 412 base-pair product from the wild-type allele. The PCR program used was 94°C for 25 seconds for one cycle; 32 cycles at 94°C for 25 seconds, 52°C for 30 seconds, 72°C for 30 seconds; and one cycle of 72°C for 5 minutes.

PCR for *rag-1* Genotypes

The *rag-1* mutation is detected by PCR amplification using a program of 94°C for 1 minute for one cycle; 32 cycles at 94°C for 30 seconds, 65°C for 30 seconds, 72°C for 3 minutes; and one cycle of 72°C for 5 minutes. The three primers used to detect the *rag-1* mutation produce a 1,993 base-pair product and a 1,549 base-pair product for the wild-type allele.

PCR for $\beta_2 m$ Genotypes

The $\beta_2 m$ mutation was detected by PCR amplification using a program of 94°C for 1 minute for one cycle; 32 cycles at 94°C for 30 seconds, 58.5°C for 30 seconds, 72°C for 3 minutes; and one cycle of 72°C for 5 minutes. A wild-type $\beta_2 m$ product was 374 base-pairs and the mutant product was 1,524 base-pairs.

PCR for *8q* Genotypes

In order to follow the fate of tetraploid cells in the diploid/tetraploid morula experiments (discussed below), fetuses and placentas were genotyped for the presence of the *8q* molecular marker. The molecular marker used was a conditional-null mutation generated by the insertion of a *lox*P/MC1-neo cassette (Deng et al., 1993) into exon B of the *Thioredoxin Reductase-1* (*TRR1*) gene on mouse chromosome 8. Mice harboring the *8q* molecular marker do not have disrupted *TRR1* gene function and are phenotypically wild-type (A. Bondareva and E. Schmidt, unpublished). The PCR reaction utilized three primers to amplify a 345 base-pair product from the wild-type allele, and a 542 base-pair product from the mutant allele. The PCR program used was 94°C for 1 minute for one cycle; 32 cycles at 94°C for 20 seconds, 61.5°C for 30 seconds, 72°C for 1 minute and 30 seconds; and one cycle of 72°C for 5 minutes.

Breeding the *tbp*$^{\Delta N}$ Mutation Into the SCID, RAG1-Null and β_2M-Null Mouse Lines

All mice in this study have been extensively backcrossed into the C57Bl/6 line, except where otherwise indicated. C57Bl/6, BALB/c and CD1 mice were purchased from Charles River Laboratories (Wilmington, MA). RAG-1- and β_2M-null mice were from Jackson Laboratories (Bar Harbor, ME). To produce *tbp*$^{\Delta N/+}$; *rag-1*$^{-/-}$ animals, studs that were wild type for TBP and homozygous for the *rag-1* mutation (*tbp*$^{+/+}$; *rag-1*$^{-/-}$) were crossed with *tbp*$^{\Delta N/+}$; *rag-1*$^{+/+}$ dams. F1 sibling crosses were used to create the F2 generation. The *tbp* mutation was bred into the β2M knockout line by crossing *tbp*$^{+/+}$;$\beta_2 m^{-/-}$ studs with *tbp*$^{\Delta N/+}$;$\beta_2 m^{+/+}$ females and F1 siblings were bred to produce F2 generation containing *tbp*$^{\Delta N/+}$;$\beta_2 m^{-/-}$ mice.

Diploid/Tetraploid Chimeric Embryo Fusions

All mice utilized for these experiments were from our BALB/c/C57Bl/6 hybrid colonies. Embryo donor females were induced to superovulate by injecting 7.5 International Units (IU) of pregnant mare's serum (PMS, Calbiochem) intraperitoneally into each animal. Ovulation was induced by injecting 5 IU of human chorionic gonadotropin (hCG, Calbiochem) per mouse 46 hours after PMS. Females were then set up 1:1 with individually housed studs and the presence of a vaginal plug on the next morning (E0.5 of pregnancy) indicated which females had successfully copulated. BALB/c/C57Bl/6 hybrid females to be used as surrogate mothers were set up with vasectomized males at the same time to induce pseudopregnancy. All embryos were cultured overnight in M16 media (Sigma) in a tissue culture incubator set at 37^{o} C/5% CO_2.

For *tbp*$^{+/+}$ BALB/c/C57/Bl6 (F1 generation hybrids) tetraploid embryos, 5-12 week old BALB/c females were crossed with *tbp*$^{+/+}$ C57Bl/6 studs harboring a molecular marker (*8q*) and embryos were harvested at E1.5 (2-cell stage) by flushing oviducts with M2 media. The 2-cell embryos were suspended in 0.3M mannitol and electro-fused to form 1-cell tetraploids using a BLS CF-150 impulse generator (Biological Laboratory Equipment, Maintenance and Service, Ltd., Budapest, Hungary) set at a DC current of 94 Volts and a square wave pulse of 25 μs (Guillemot et al., 1994; Nagy et al., 1990). Tetraploid embryos were cultured overnight to form 4-cell morulae (Fig. 7).

Diploid embryos were generated from *tbp*$^{\Delta N/+}$ BALB/c/C57Bl/6 females

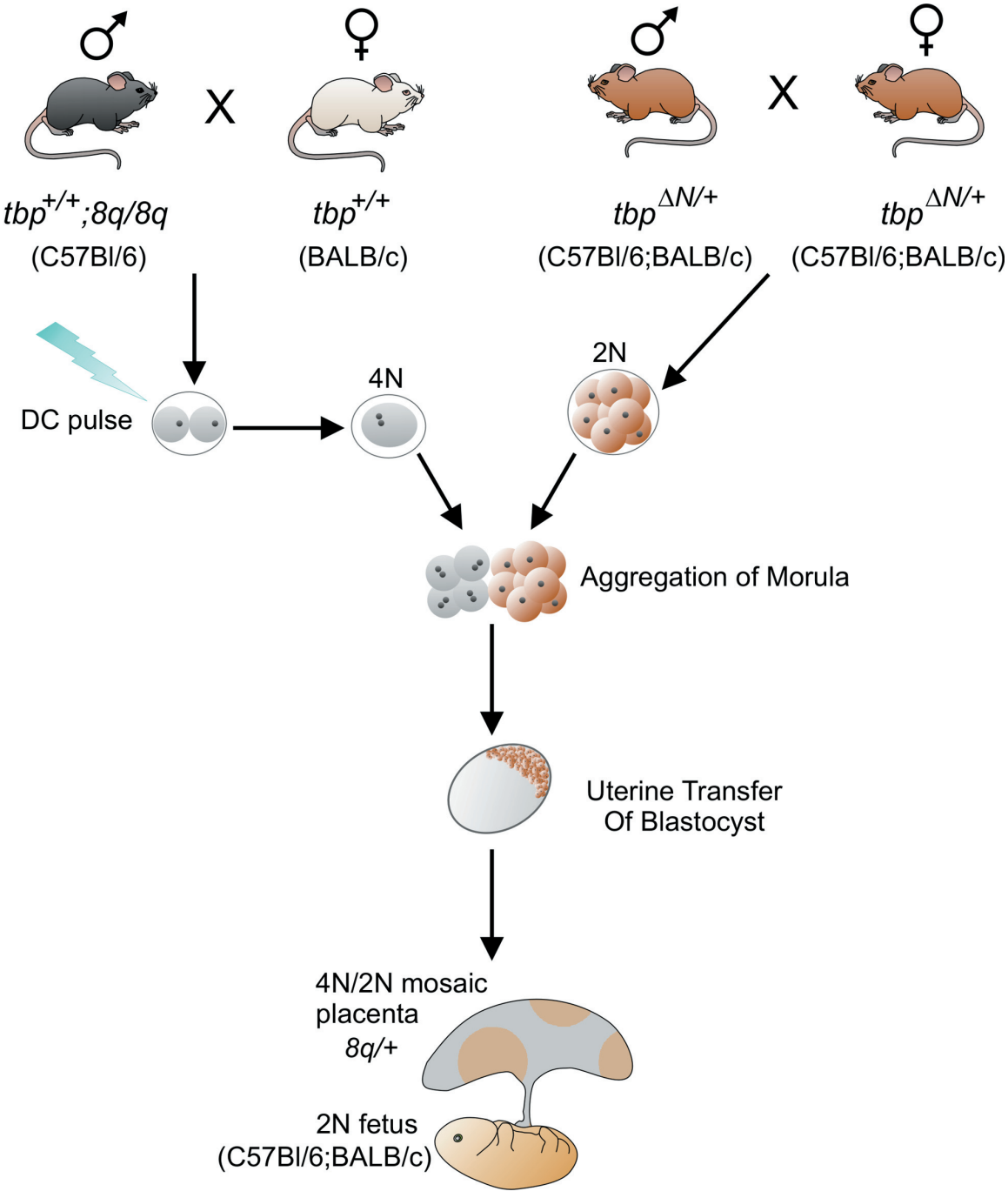

Figure 7. Flow chart depicting the process of generating diploid fetuses and diploid/tetraploid placentas. Tetraploid cells and tissues are gray. Diploid cells and tissues are brown. Tetraploid cells are followed by assaying for the presence of the 8q marker gene.

crossed to *tbp*$^{\Delta N/\Delta N}$ Balb/c/C57Bl/6 males. E2.5 morulae (8-cell stage) were harvested by flushing uteri and oviducts with M2 media. The zonae of both 4-cell tetraploid and diploid morulae was removed using Acid Tyrode's solution (Sigma) and the morulae were cocultured overnight to form chimeric embryo aggregates. The next day, chimeric blastocysts were transferred to the uteri of E2.5 pseudopregnant *tbp*$^{+/+}$ Balb/c/C57/Bl6 (F1) females (in mice, pseudopregnancy is induced by the mechanical stimulation of the uterine cervix during mating, resulting in temporary hormonal changes that are similar to those in actual pregnant females (Chambers and Clarke, 1979). Fetuses were birthed by Cesarean section on E18.5, small fetal and placental biopsies were taken for genotypic analyses, and pups were fostered to CD1 mothers with age-matched litters. Pup and placenta samples were analyzed for *tbp* genotypes and presence of the *8q* molecular marker. Placentas from non-*tbp*$^{+/+}$ pups were considered to be positive for tetraploidy if they were both *tbp*$^{+/+}$ and heterozygous for the *8q* molecular marker. Placentas that were associated with *8q* -positive pups were not counted in further analyses. Pups from these experiments were genotyped at 1 week of age to determine *tbp* genotype ratios.

CHAPTER 3

THE MIDGESTATIONAL CRISIS

Introduction

Previous Work from Our Group

TBP in mammals is composed of a core domain that is shared with all eukaryotes and an N-terminal domain that is specific to vertebrates. Given the high level of conservation of the TBP N terminus in vertebrates, we have hypothesized that this domain could represent a new surface on the basal transcription machinery that has coevolved with regulators to coordinate greater genetic complexity in mammals. To test this, a targeting vector in *tbp* was designed to replace the wild type allele in mice. The resulting mutant allele, $tbp^{\Delta N}$, resulted in a protein lacking 111 amino acids of the 135 amino acid vertebrate-specific N-terminal domain. It was predicted that this mutation would create a defect in a gene pathway(s) required in vertebrates.

The N Terminus is not Required in Basal Processes

TBP is a requisite component of the basal transcription machinery of eukaryotes. Although animals bearing the $tbp^{\Delta N}$ mutation were viable (discussed in detail below), it was possible that the mutation compromised cellular functions in a sublethal manner. For example, it is possible that physiological abnormalities do exist in $tbp^{\Delta N/\Delta N}$ fetuses, but these defects alone do not affect essential systems during gestation. To test these possibilities, our group generated primary mouse embryo fibroblast cell lines (MEFs) derived from $tbp^{+/+}$,

tbp$^{\Delta N/+}$, and *tbp*$^{\Delta N/\Delta N}$ fetuses. Multiple cell lines of each genotype were examined and found to be indistinguishable in terms of survival, cell proliferation rates, transcription initiation site fidelity by RNA polymerase II and III, and splicing efficiency (Schmidt et al., 2003). The cell cultures were examined for differences in levels of mRNA of the S phase-specific marker *dihydrofolate reductase* (*DHFR*), and the G_2-specific marker, *cdc25B*. Importantly, all cell lines had essentially the same proliferation rates, suggesting that no genotype-dependent differences existed. Levels of U6 RNA, a pol III-dependent component of the splicing machinery, housekeeping genes from both TATA-containing and TATA-less promoters, and global gene expression were also examined and found to be the same across all *tbp* genotypes (Schmidt et al., 2003). Likewise, levels of total TBP protein from all three MEF genotypes were similar, as determined by Western blot analysis. This data indicated that the TBP N terminus does not likely participate in general cellular functions, unless these functions can be compensated for by redundant systems.

tbp$^{\Delta N/\Delta N}$ Fetuses Die at Midgestation

Gestation in a C57Bl/6 mouse is approximately E19.3 +/- 0.5d. Litters from heterozygous crosses were harvested each day starting from E8.5 through E13.5. Mendelian ratios of genotypes were observed up to E9.5 and all fetuses appeared normal (Hobbs et al., 2002). At this time, resorbing fetuses, assumed to be mutants, were present along with live mutant fetuses that appeared normal, albeit somewhat runted on many occasions. Examination of intact homozygous fetuses did not reveal defects in the ability of cells to survive, proliferate, differentiate or assemble into tissues and organs. The fetuses had developed organ systems, beating hearts and circulating blood. Litters harvested after E13.5 contained approximately 11% of the expected number of *tbp*$^{\Delta N/\Delta N}$ fetuses.

Most fetuses homozygous for the *tbp*$^{\Delta N}$ mutation die between E10.5 and E12.5, and we have called this period the midgestational crisis. After the midgestational crisis, no additional loss of *tbp*$^{\Delta N/\Delta N}$ fetuses was observed during gestation. When litters from *tbp*$^{\Delta N/+}$ crosses were examined at weaning, less than 1% of the expected number of *tbp*$^{\Delta N/\Delta N}$ pups were alive. Thus, loss of the remaining 11% of *tbp*$^{\Delta N/\Delta N}$ animals occurred between E17.5 and weaning (3 weeks of age) (Hobbs et al., 2002). This later crisis will be addressed in Chapter 4. Those *tbp*$^{\Delta N/\Delta N}$ mice that did survive to adulthood appeared healthy and possessed normal fertility (Schmidt et al., 2003). These observations suggested that the *tbp*$^{\Delta N}$ mutation was not likely toxic. *tbp*$^{\Delta N/\Delta N}$ adults did not exhibit a higher frequency of rearing *tbp*$^{\Delta N/\Delta N}$ pups, indicating that their survival is not due to a heritable trait. We did not observe any particular mothers to be more tolerant of their mutant offspring, suggesting that maternal determinants did not play a role in *tbp*$^{\Delta N/\Delta N}$ fetal survival (Hobbs et al., 2002).

tbp$^{\Delta N/\Delta N}$ Fetus Survival is Not Due to Heritable Traits

Most *tbp*$^{\Delta N/\Delta N}$ fetuses died in the midgestation crisis. However, the few that survived to adulthood did not exhibit fertility defects and appeared normal and healthy. When we bred surviving *tbp*$^{\Delta N/\Delta N}$ mice together, we did not exhibit an increased incidence of rearing *tbp*$^{\Delta N/\Delta N}$ pups. This suggested that survival of some *tbp*$^{\Delta N/\Delta N}$ fetuses was not a result of a heritable genetic trait.

Haplotype Differences

Haploytype, the set of MHC alleles that an individual inherits, generally plays a central role in the mechanisms of MHC I-mediated rejection. The *tbp* gene is on chromosome 17 and is tightly linked (<10 cMorgans) to the MHC complex. Our *tbp*$^{\Delta N}$ mutation was first generated in embryonic stem (ES) cells from 129X1 mice, which are haplotype b (Margulies, 1999). Thus, nearly all of

the *tbp*$^{\Delta N}$ alleles in these studies carried with them a 129X1 haplotype b MHC complex. Our mice were then extensively backcrossed into C57Bl/6 mice. Because C57Bl/6 mice are also haplotype b, all mice in these studies, whether *tbp*$^{\Delta N/+}$ or *tbp*$^{\Delta N/\Delta N}$, were haplotype b. This means that haplotype differences does not likely play a role in rejection of *tbp*$^{\Delta N/\Delta N}$ fetuses.

The Placentas of *tbp* Mutants

The placenta is the vital, transient organ that allows the embryo to implant into the uterus for gas and nutrients exchange between the developing fetus and mother. To do this, the placenta promotes growth of maternal blood vessels into the implantation site while suppressing the local maternal immune system (Cross et al., 2002). Because we did not observe defects in *tbp*$^{\Delta N/\Delta N}$ fetuses that could account for their death at midgestation, we considered that *tbp*$^{\Delta N/\Delta N}$ placentas may not be functioning properly, or perhaps are not interacting with the maternal immune system appropriately. Placentas examined after E12.5 revealed signs of histolysis. Microscopic analysis of *tbp*$^{\Delta N/\Delta N}$ placentas occasionally revealed embryonic red blood cells mixing with maternal blood. Because no other phenotype other than a defect in the placenta is observed when the N terminus of TBP is removed in mice, we hypothesized that *tbp*$^{\Delta N/\Delta N}$ fetuses die at midgestation due to placental defects. To test this, we generated *tbp*$^{\Delta N/\Delta N}$ fetuses on *tbp*$^{+/+}$ tetraploid placentas via chimera generation. The results of these experiments indicated that those *tbp*$^{\Delta N/\Delta N}$ fetuses that had been supplied a *tbp*$^{+/+}$ tetraploid placenta survived midgestation (Hobbs et al., 2002). These results supported our hypothesis that *tbp*$^{\Delta N/\Delta N}$ fetuses die at midgestation due to placental defects.

Results

Fetal Rejection is Not Due to Classical Immune Responses

Classical adaptive immune responses result in a state of immunological memory where the immune system responds more rapidly and effectively to antigens encountered previously, reflecting the pre-existence of a clonally expanded population of antigen-specific lymphocytes (Bousso and Kourilsky, 1999). If death of *tbp*$^{\Delta N/\Delta N}$ fetuses was due to a classical adaptive immune response, we would expect mothers to reject their mutant fetuses more effectively the second time they carried them. Thus, all surviving *tbp*$^{\Delta N/\Delta N}$ animals would be expected to come from first time mothers only. After examining the maternal histories of surviving *tbp*$^{\Delta N/\Delta N}$ adults, we found no correlation between homozygote survival and the mothers they came from. Additionally, we do not have any evidence that rejection of one *tbp*$^{\Delta N/\Delta N}$ placenta necessarily lead to rejection of another mutant placenta in the same pregnancy. This suggests that the maternal immune response against mutant placentas is a localized event. Taken together, these observations do not support the likelihood that death of *tbp*$^{\Delta N/\Delta N}$ fetuses involve a classical MHC I-mediated immune response.

Rescue of *tbp*$^{\Delta N/\Delta N}$ Fetuses in Immunocompromised Females

Breeding into SCID Mice

tbp$^{\Delta N/\Delta N}$ fetuses begin dying at approximately E10.5, around the same time that the mature placenta is established and fetal tissues, along with fetal antigens, are coming into close contact with maternal tissues. Because we observed a placental defect in homozygous mutants, we considered that this

defect could be stimulating a maternal immune response, resulting in death of mutants at midgestation. To test this, we crossed *tbp*$^{\Delta N}$ mice with the immunocompromised lines, Severe Combined Immunodeficiency (SCID) and recombination activating gene -1 (RAG-1) knockout mice. We hypothesized that if *tbp*$^{\Delta N/\Delta N}$ fetuses are the subject of a maternal immune response, rejection would be diminished in immunocompromised mothers.

We first bred the *tbp* mutation into SCID mice. The *scid* mutation was first discovered by Bosma et al. in 1983 (Bosma et al., 1983), and was later found to be comprised of a single T to A nucleotide transversion within the region encoding the catalytic subunit of a DNA-dependent protein kinase (DNA-PKcs) (Araki et al., 1997; Blunt et al., 1996). As a result, SCID mice are deficient in the ability to rearrange the elements encoding immunoglobulin and T-cell receptor genes, and lack mature T and B lymphocytes. The F1 generation was produced by breeding studs that were homozygous for *tbp*$^{\Delta N}$ and wild type for the *scid* mutation (*tbp*$^{\Delta N/\Delta N}$;*scid*$^{+/+}$) to dams that were wild type for *tbp* and homozygous for the *scid* mutation (*tbp*$^{+/+}$;*scid*$^{-/-}$). The F2 generation was made via F1 sibling crosses.

The *tbp* allele can be followed via PCR. Because the *scid* mutation is a single base nonsense mutation, however, it cannot be followed via PCR with accuracy. Instead, other methods have been generated to follow this mutation. One reported method takes advantage of the *Alu I* restriction site created by the *scid* mutation (Blunt et al., 1996) in exon 85 of the DNA-PKcs. First, this region is amplified by PCR and then the PCR product is cut with Alu I. By assaying the results of this restriction reaction via gel electrophoresis, animals that possess the *scid* mutation (a cleaved PCR product) should be distinguishable from wild type animals (intact PCR product). However, due to the possibility of failed or

partial *Alu I* digests, a mutant allele could be misidentified as wild type. A more classical approach takes advantage of the absence of mature B cells in SCID mice and assays for a SCID phenotype: the presence or absence of serum IgG. Initially, for the studies discussed below, this method was used to follow the *scid* mutation. Animals that had immunoreactive serum with an anti-mouse IgG antibody were considered to be carrying a wild type *scid* allele. When serum did not exhibit immunoreactivity with anti-mouse IgG antibody, the animals from which these samples came were considered to be $scid^{-/-}$. However, $scid^{+/-}$ animals could not be readily distinguished from $scid^{+/+}$ animals by this method because the *scid* mutation is recessive. Athough the genotypes of these animals could be retroactively determined through specific breedings, this method would take many months to perform. Thus, our group sought to design a genotyping method that was rapid and accurate. A member of our group, Amy Sealey, developed a molecular method to follow the *scid* allele: a region of the DNA-PK(cs) is amplified via PCR and the PCR products are directly sequenced. In this way, we were able to rapidly identify between $scid^{+/-}$ and $scid^{+/+}$ animals (Fig. 8) (Sealey et al., 2001).

To test whether $tbp^{\Delta N/\Delta N}$ fetuses could survive in SCID mothers, we harvested E13.5 fetuses from $tbp^{\Delta N/+}$;$scid^{-/-}$ dams that had been bred to $tbp^{\Delta N/+}$;$scid^{+/+}$ studs. From 6 litters, we found $tbp^{+/+}$: $tbp^{\Delta N/+}$: $tbp^{\Delta N/\Delta N}$ ratios to be 21:26:10 (Table 1). Thus, we observed a 47% rescue of $tbp^{\Delta N/\Delta N}$ fetuses in the $tbp^{\Delta N/+}$;$scid^{-/-}$ dams, which was almost 4-fold over the survival rate in strain-matched control wild-type dams (Table 1).

Breeding into the *rag-1* Mutation

Although our initial results from breeding into SCID mice appeared to

Table 1. Fetal Survival Past Midgestation in Immunocompromised Mothers[a]

Maternal Genotype[b]	*tbp*$^{+/+}$	*tbp*$^{\Delta N/+}$	*tbp*$^{\Delta N/\Delta N}$	n (litters [ave. size])
tbp$^{\Delta N/+}$;*scid*$^{+/+}$	15 (100%)	28 (93%)	2 (13%)[c]	45 (7 [8.1])
tbp$^{\Delta N/+}$;*scid*$^{-/-}$	21 (100%)	26 (62%)	10 (47%)	57 ($p < 0.001$)[d] (6 [7.5])
tbp$^{\Delta N/+}$;*rag*$^{-/-}$	13 (100%)	30 (115%)	16 (123%)	59 ($p < 0.001$)[d] (7[8.4])

[a]Adapted from Supplemental Table S1 from Hobbs, *et al*., 2002.
[b]All females were crossed to *tbp*$^{\Delta N/+}$ males.
[c]Percent survival based on expected Mendelian ratios and 100% survival of *tbp*$^{+/+}$ fetuses.
[d]Chi-square test demonstrating that genotype ratios differ significantly from *tbp*$^{\Delta N/+}$ X *tbp*$^{\Delta N/+}$ matings, $\alpha = 0.05$.

support the hypothesis that diminishing the maternal immune response would rescue *tbp*$^{\Delta N/\Delta N}$ fetuses, we had two concerns about using these animals. First, the mice that harbor the *tbp* mutation are on a C57Bl/6 background while SCID mice are on strain C.B-17 (a congenic descendent of BALB/c) (Bosma et al., 1983). On chromosome 17 at the MHC complex, C.B-17 mice are haplotype d (Margulies, 1999). On the other hand, C57Bl/6 mice are haplotype b (Margulies, 1999). Therefore, by breeding the *tbp* mutant mice to the SCID mice, we generated a hybrid F1 generation that is on the mixed background C57Bl/6/BALB/c (haplotype d/b). Thus, these animals were no longer inbred, but possessed chromosomal heterozygosity (Eggan et al., 2001) at every locus of the genome. Because of this, we could not directly compare results of *tbp*$^{\Delta N/\Delta N}$ fetal survival in *tbp*$^{\Delta N/+}$;*scid*$^{-/-}$ mothers with survival in mice on pure C57Bl/6 backgrounds. Second, the *scid* mutation in some young mice and virtually all

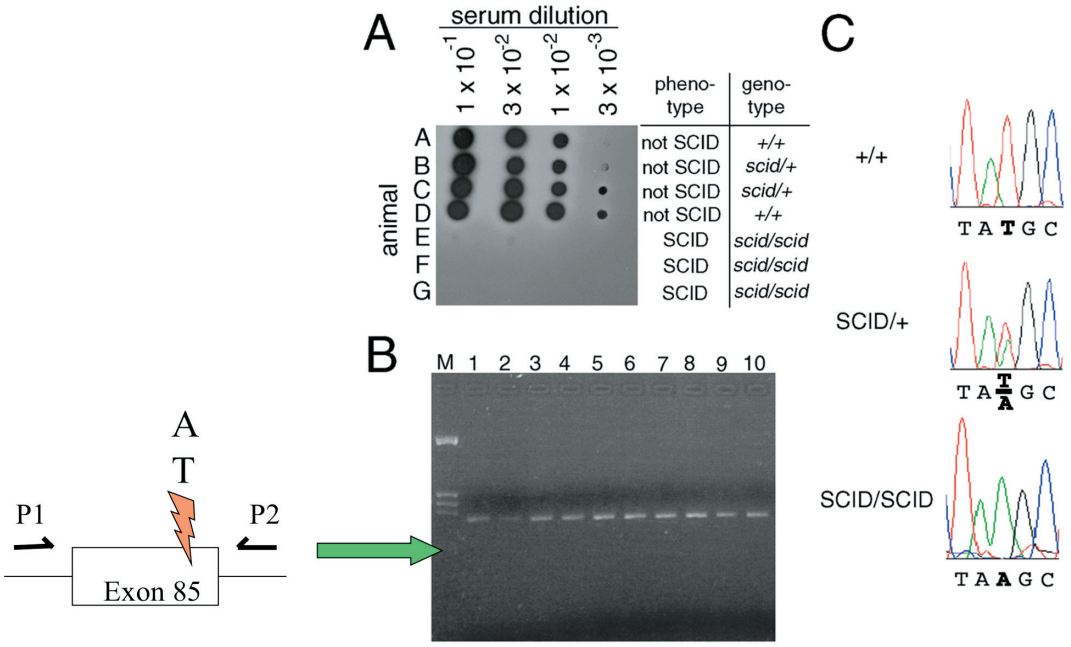

Figure 8. Methods for genotyping the *scid* mutation in mice. A. Serum assay for the presence or absence of IgG from serum of mice. Note that a difference cannot be easily determined between $scid^{+/+}$ and $scid^{+/-}$ animals. B. Electrophoretic gel with PCR products of a region of the DNA-PK(cs). C. Results from directly sequencing PCR products from (B). A "T" at position at Tyr-4046 in exon 85 indicates the wild-type allele and an "A" represents the mutant allele.

old SCID mice has shown to be "leaky", meaning it allows the mice to regain the ability to express functional antigen receptor (Bosma et al., 1988; Carroll and Bosma, 1988; Carroll et al., 1989). This leakiness is partly due to reversion of the point mutation responsible for the SCID phenotype in individual lymphocyte progenitors (Petrini et al., 1990). To address these issues and to confirm that $tbp^{\Delta N/\Delta N}$ fetuses fail because of a maternal immune response, we repeated these experiments using the rag-1 knockout line of mice (Hobbs et al., 2002). The rag-1-null line is on a C57Bl/6 (haplotype b) background and no leakiness has been reported for the mutation (Mombaerts et al., 1992).

F1 and F2 generations were created by crossing studs that were wild type

for *tbp* and homozygous for the *rag-1* mutation ($tbp^{+/+}$;$rag^{-/-}$) with $tbp^{\Delta N/+}$; $rag^{+/+}$ dams. F1 sibling crosses were used to create the F2 generation. Both alleles were followed via PCR. We harvested fetuses at E13.5 from $tbp^{\Delta N/+}$;$rag^{-/-}$ dams that had been crossed to $tbp^{\Delta N/+}$ $rag^{+/+}$ studs. From these matings, 100% of $tbp^{\Delta N/\Delta N}$ fetuses survived the midgestational crisis (Table 1). These results supported our hypothesis that $tbp^{\Delta N/\Delta N}$ fetuses succumb to a maternal immune response. Together, the breeding of $tbp^{\Delta N/+}$ animals with immunocompromised mouse lines led to two conclusions at this point. First, since $tbp^{\Delta N/\Delta N}$ fetuses survive the midgestational crisis in immunocompromised mothers, it was unlikely that instrinsic factors in $tbp^{\Delta N/\Delta N}$ placentas alone are the cause of $tbp^{\Delta N/\Delta N}$ fetal death at midgestation. Second, $tbp^{\Delta N/\Delta N}$ fetal death at midgestion was likely the result of a maternal immune response.

The $\beta_2 m$ Mutation – Addressing the Potential for a Maternal Immune Response

At this point, we had observed a defect in $tbp^{\Delta N/\Delta N}$ placentas in immune-normal mothers, and rescue of $tbp^{\Delta N/\Delta N}$ fetuses at midgestation was obtained in immunocompromised mothers. We next considered whether removal of the TBP N terminus resulted in maternal immune rejection because of inappropriate fetal/placental antigen exposure. It is well established that the trophoblast cells of placenta represent an interface where recognition of fetal antigens, presented in MHC molecules, by effector mechanisms take place. Trophoblast cells are thought to be the site of immune regulation by complex cellular and molecular interactions (Clark, 1991). Thus, antigens present at this interface likely play keys roles in the feto-maternal immunological relationship throughout gestation (reviewed in Szekeres-Bartho, 2002). We considered that if fetal-derived antigen

Table 2. Fetal Survival Past Midgestation as a Function of Fetal β_2M Genotype[a]

Fetal β_2M Genotype	*tbp*$^{+/+}$	*tbp*$^{\Delta N/+}$	*tbp*$^{\Delta N/\Delta N}$	n
$\beta_2M^{+/+}$	25 (100%)	42 (84%)	2 (8%)[b]	69
$\beta_2M^{-/-}$	29 (100%)	55 (95%)	14 (48%)	98 ($p < 0.001$)[c]

[a]All fetuses were from *tbp*$^{\Delta N/+}$;$\beta_2M^{+/-}$ females crossed to males that were *tbp*$^{\Delta N/+}$;$\beta_2M^{+/+}$, *tbp*$^{\Delta N/+}$;$\beta_2M^{+/-}$, or *tbp*$^{\Delta N/+}$;$\beta_2M^{-/-}$ males.
[b]Percent survival based on expected Mendelian ratios and 100% survival of *tbp*$^{+/+}$ fetuses.
[c]Chi-square test demonstrating that *tbp* genotype ratios of $\beta_2M^{-/-}$ fetuses differ significantly from those of $\beta_2M^{+/+}$ fetuses, $\alpha = 0.05$.

from the *tbp*$^{\Delta N/\Delta N}$ fetus/placenta triggers a lethal maternal immune response, then blocking MHC I placental expression may rescue them. To test this, we chose to breed our *tbp* mutation into a mouse line deficient for β_2M, the molecule required for proper MHC I cell surface expression. This line was chosen because a simple MHC knock-out mouse would not be useful for our purposes: there are 4 classical MHC I genes, and potentially 100 or more non-classical MHC I genes in the mouse genome. We generated *tbp*$^{\Delta N/+}$;$\beta_2M^{+/-}$ females and bred them to *tbp*$^{\Delta N/+}$;$\beta_2M^{+/+}$, *tbp*$^{\Delta N/+}$;$\beta_2M^{+/-}$, or *tbp*$^{\Delta N/+}$;$\beta_2M^{-/-}$ males. From these crosses, we found that only 8% of those *tbp*$^{\Delta N/\Delta N}$ fetuses that were $\beta_2M^{+/+}$ survived mid-gestation (Table 2). In contrast, those *tbp*$^{\Delta N/\Delta N}$ fetuses that were simultaneously homozygous for the β_2M mutation ($\beta_2M^{-/-}$), survived 6-fold (48% survival). This data suggested that the placental defect we observe in *tbp*$^{\Delta N/\Delta N}$ placentas involves a β_2M–dependent process in the placenta/fetus. Thus, the *tbp*$^{\Delta N/\Delta N}$ condition may affect the way in which antigens are regulated and/or are appropriately expressed in the presence of the maternal immune system.

Conclusion/Discussion

Immune System Disruption and *tbp*$^{\Delta N/\Delta N}$ Fetus Survival

Although our mice are all syngeneic, or genetically identical, (with the exception of the SCID mice experiments), it is possible that *tbp*$^{\Delta N/\Delta N}$ fetuses/ placentas encounter rejection from expression of endogenous antigens that are not recognized as self by the maternal immune system. There are many reports of such antigens in other parts of the body including those from cancerous cells (Schreiber, 1999) and from immune-sequestered tissues like cornea (Shevach, 1999) and testis (Hall et al., 1994). Another source of endogenous antigen that may not be recognized by the maternal immune system could come from fetal tissue- or stage-specific genes (Wegmann et al., 1979). Still another potential source of antigens comes from a reported retrovirus. Syncytin is a protein encoded by a known retrovirus in placenta that is required for proper development of trophoblast giant cells (Mi et al., 2000) Thus, removing the N-terminus of TBP has could potentially disrupt mechanisms by which these "foreign" antigens either remain sequestered from the maternal immune system or are otherwise regulated for appropriate expression.

We have generated a mutation in a component of the basal transcription machinery that has resulted in a tissue- or situation-specific defect: rejection of homozygous mutants at midgestation. The maternal immune system must remain competent through pregnancy for defense against microbial infections, while refraining from mounting a response against the fetus, which represents a foreign tissue to the mother. It is now well-documented that polymorphic MHC I antigen presentation appears to be absent on the surface of some trophoblast

cells. This has shown to be the case for endovascular trophoblasts that are in direct contact with maternal blood cells (Coady et al., 1999; Drezen et al., 1992; Hunt and Orr, 1992; Jaffe et al., 1990; Jaffe et al., 1991; Oudejans et al., 1989). The absence of MHC I antigen presentation, however, does not appear to be due to a lack of expression of MHC I in these cells. On the contrary, studies reveal that the outer trophoblast layer of the placenta do express MHC I, but they lack the simultaneous expression of β_2M (Jaffe et al., 1991). Because expression of antigen to $CD8^+$ T cells requires the accompaniment of β_2M, the sequestering of β_2M in some trophoblast cells may be a mechanism by which the fetus avoids a maternal immune response. This concept is consistent with the idea that the outer zone of trophoblast giant cells function as an immunologic barrier.

The non-coordinate regulation of β_2M and MHC I in cell layers of the placenta may be indicative of a delicate balance of regulation, dictating the expression of fetal-derived antigen presentation. Because *tbp*$^{\Delta N/\Delta N}$ fetuses survive midgestation when they lack β_2*m* expression or when maternal T and B cell components are eliminated via the *rag-1* (or *scid*) mutation, it is possible the TBP N terminus is required in this delicate balance to orchestrate the expression (or suppression) of β_2*m* and/or *MHC I*. Without the N terminus, inappropriate fetal antigen presentation, either spatially or temporally, may occur, leading to a maternal immune response. Alternatively, it is also possible the *tbp*$^{\Delta N}$ mutation leads to inappropriate expression of certain antigens themselves that, unless suppressed by the removal of β_2M or maternal adaptive immunity, are immunogenic and can trigger an immune response.

A Role for Nonclassical/ MHC-I-like molecules and NKT cells?

Classical MHC I molecules requires assembly with β_2M for cell surface

expression. Many non-classical MHC I molecules are also β_2M-dependent and have been reported to play a role in rejection responses as well. Unlike classical class I genes, the nonclassical class I *MHC* genes are encoded by the murine *T*, *Q*, and *M* regions (Shawar et al., 1994) and exhibit little or no polymorphism. This lack of diversity among these genes suggests that the nonclassical genes do not likely have the ability to bind and present a broad array of antigenic peptides the way the classical MHC I do (Margulies, 1999). In general, they also have a more restricted expression pattern than classical class I MHC products and are usually expressed at lower levels (Margulies, 1999). Although the function of some of these nonclassical class I molecules is unknown, one study has revealed a role in placental rejection for a non-CD1, MHC-I-like, molecule that is β_2M-dependent and present on placenta. These molecules can mediate rejection of the placenta via stimulation of a subset of T cells called Vα14 NKT cells (Dang and Heyborne, 2001). Thus, disruption in expresstion of β_2M-dependent nonclassical molecules could also play a role in increased survival of *tbp*$^{\Delta N/\Delta N}$ fetuses at midgestation.

Removal of β_2m May Affect Other Systems

Some nonclassical MHC Ib molecules have functions that are not directly related to the immune system. The MHC class I family-like Fc receptor, FcRn, is normally responsible for extending the life span of serum IgG (Akilesh et al., 2004) and neonatal FcRn controls homeostasis and fetal/maternal transfer of serum gamma-globulins (Dall' Acqua et al., 2002). FcRn was originally described in rat and was reportedly located within the intestinal epithelium to function in transport of colostral immunoglobulin from the lumen to the bloodstream (Simister and Rees, 1985). Homologues of FcRn have been found in both mouse and

humans. Animals that lack β_2M seem to metabolize serum immunoglobulin inappropriately due to a lack of cell surface expression of FcRn (Israel et al., 1996). Further, β_2M appears to be required for cell surface expression of FcRn: in the absence of β_2M, FcRn is retained in the endoplasmic reticulum and IgG binding is decreased compared with that of native FcRn (Praetor and Hunziker, 2002). Taken together, these studies demonstrate that removal of β_2M from our mice in an attempt to rescue *tbp*$^{\Delta N/\Delta N}$ fetuses may, in fact, have affected systems other than just the adaptive immune system. However, given the results of our *rag*$^{-/-}$ mice studies, it is likely that β_2M-deletion in *tbp*$^{\Delta N/\Delta N}$ fetuses increases survival at the midgestational crisis, at least in part, because it eliminates inappropriate interactions with the maternal immune system.

Other Functions of MHC I

There have been some reports that suggest MHC class I glycoproteins possess a broader biological significance outside of their role in cell-surface antigen presentation on self-cells (Assa-Kunik et al., 2003). In one paper, syngeneic misexpression of an H-2D^d transgene in embryos was shown to lead to placental failure. The authors of this study concluded that the mechanisms of placental failure were not likely immune-mediated because transfer of transgenic embryos into a syngeneic surrogate mother could not rescue them. Instead, it was suggested that embryonic lethality could have occurred via disruption of other essential functions perturbed by missexpression of the H-2D^d transgene (Jaffe et al., 1992). These glycoproteins were further implicated in non-immune functions as studies revealed roles in regulation of proliferation and apoptosis in lymphoid and nonlymphoid cells (Huh et al., 2000; Pedersen et al., 1999). It has been demonstrated, as well, that MHC I molecules can form complexes with

other membrane-bound molecules including transmembrane receptors for growth factors and cytokines (Matko et al., 2002). Among these receptors are those for insulin, insulin-like growth factor (IGF), epidermal growth factor, and various other factors (Cremaschi and Sterin-Borda, 1994). Peptides from the α1 domain of MHC class I have been shown previously to augment glucose uptake in cells when associated with epidermal growth factor (EGF) (Stagsted et al., 1993). This same peptide inhibits the internalization of glucose transporters (GLUT4), insulin-like growth factor 1 (IGF-1) receptors, and insulin receptors on the cell surface which increases glucose uptake by the cell (Stagsted et al., 1993a). Given these functions, it is not surprising that these peptides can also cause hypoglycemia in rats (Stagsted et al., 1990). Thus, these receptors function in pathways that, when disrupted, could lead to the embryonic lethality we observe in our *tbp*$^{\Delta N/\Delta N}$ fetuses. Further, removal of β2M could potentially eliminate the aberrant activity of MHC I in such cases.

The Danger Model

The function of the immune system has classically been defined as having the ability to discriminate between self and nonself, tolerating self and attacking nonself (Margulies, 1999). Recently, a model was put forth suggesting that the maternal immune response can be triggered by endogenous distress signals rather than by nonself antigens (Matzinger, 2002). Called the “danger” model of immunity, this model suggests that the immune system is more concerned with damage than with foreignness, responding to the presence of alarm signals from injured tissues, rather than from nonself recognition. The “danger model” is intriguing because it is consistent with many of our observations. All of our mice are on an inbred C57Bl/6 background and are syngeneic. We are able to rescue

tbp$^{\Delta N/\Delta N}$ fetuses at midgestation by either ablating the maternal immune response or by disrupting fetal/placental β_2*M* expression (Hobbs et al., 2002). Because we observe a defect in some *tbp*$^{\Delta N/\Delta N}$ placentas, it is possible that, on the cellular level, a distress signal is being sent. Thus, perhaps in removing the TBP N terminus, we have made a "sick" placenta, that sends a danger signal into the milieu to the maternal immune system.

In these studies, we have examined a placental defect in mice that was generated by removing a vertebrate-specific protein domain, the TBP N terminus. Most homozygous mutants die at midgestation from an apparent defect in their placentas. Mutant fetuses could be rescued at this midgestational crisis if supplied with a wild-type tetraploid placenta and survived in immune-normal mothers when fetal/placental β_2*m* expression was genetically disrupted. Rearing mutants in immunocompromised mothers are rescued at midgestation. These results suggest the N terminus of TBP functions in β_2M-dependent processes and within the placenta to favor immunotolerance during pregnancy at midgestion.

We have posited that the N terminus of TBP in mice participates in gene regulatory events that ultimately protect the fetus from maternal immune rejection processes. While other functions for the N terminus likely exist, many of them may have had time in evolution to be "backed-up" by redundant systems, such as those involving other *tbp* family members. Thus, the placental defect we observe in mice may be the result of disrupting TBP pathways that have arisen recently in vertebrate evolution.

CHAPTER 4

THE LATE CRISIS

Introduction

After implantation, the hemochorial placenta forms so that physiological exchanges of nutrients, gas, and waste can take place between maternal and fetal tissues. The placenta secretes hormones (reviewed in Makrigiannakis et al., 2003; Soares, 2004) along with other agents to regulate immune pathways at the fetomaternal interface and to targeting maternal physiological systems (Cross et al., 2003a) that lead to successful pregnancy.

In general, the birth of healthy offspring is dependent upon normal placental development and function. Conversely, abnormal placentation is responsible for a wide range of pregnancy-related complications, including miscarriage, preeclampsia, intrauterine growth retardation, and placental abruption (premature detachment from the uterus before birth) (Kingdom et al., 2000).

In immunocompetent C57Bl/6 mice, >90% of *tbp*$^{\Delta N/\Delta N}$ fetuses die at mid-gestation. Our data indicate that from E17.5 of gestation to weaning (3 weeks post-natal), another crisis occurs which eliminates approximately 67% of the remaining mutants (Hobbs et al., 2002). Because so few *tbp*$^{\Delta N/\Delta N}$ fetuses survive mid-gestation, it was not initially possible to address this later crisis in immunocompentent females. However, *tbp*$^{\Delta N/\Delta N}$ fetuses in *tbp*$^{\Delta N/+}$;*rag*$^{-/-}$ females survive through midgestation. Additionally, like litters from immune-normal females, litters from *tbp*$^{\Delta N/+}$;*rag*$^{-/-}$ females contained almost no surviving *tbp*$^{\Delta N/\Delta N}$ pups at weaning. Thus, *tbp*$^{\Delta N/+}$;*rag*$^{-/-}$ females served as a good model in which to

Table 3. Fetal Survival to Weaning

Maternal Genotype[a]	*tbp*$^{+/+}$	*tbp*$^{\Delta N/+}$	*tbp*$^{\Delta N/\Delta N}$	n (litters [ave. size])
tbp$^{\Delta N/+}$;*rag*$^{-/-}$	104 (100%)	106 (51%)	1 (0.96%)[b]	211 (p < 0.0001) (42 [5.2])

[a]All females were crossed to either *tbp*$^{\Delta N/+}$;*rag*$^{-/-}$ or *tbp*$^{\Delta N/+}$;*rag*$^{+/+}$ males. No differences in survival were observed between *rag*$^{+/-}$ *or rag*$^{-/-}$ pups.
[b]Percent survival based on expected Mendelian ratios and 100% survival of *tbp*$^{+/+}$ fetuses.
[d]Chi-square test demonstrating that genotype ratios differ significantly from *tbp*$^{\Delta N/+}$ X *tbp*$^{\Delta N/+}$ matings, $\alpha = 0.05$.

study fetal death past midgestation. We found that most *tbp*$^{\Delta N/\Delta N}$ fetuses survived through gestation in *tbp*$^{\Delta N/+}$;*rag*$^{-/-}$ females, although they were runted and died shortly after birth.

Results:

Fostering Does Not Increase Survival of *tbp*$^{\Delta N/\Delta N}$ Pups

Although *tbp*$^{\Delta N/\Delta N}$ fetuses survive midgestation in *tbp*$^{\Delta N/+}$;*rag*$^{-/-}$ females, by weaning almost none have survived (Table 3). Because mutant pups were absent by weaning, we aimed to find the missing *tbp*$^{\Delta N/\Delta N}$ animals by examining the cages of *tbp*$^{\Delta N+}$;*rag*$^{-/-}$ females after birth. Thus, at some time between E17.5 and weaning, surviving *tbp*$^{\Delta N/\Delta N}$ animals die. It was expected that if *tbp*$^{\Delta N/\Delta N}$ fetuses survived to birth in *tbp*$^{\Delta N+}$;*rag*$^{-/-}$ females, we may be able to find them by searching cages after birth. When we did this, we occasionally found partial or whole pups. However, the amount of pups recovered did not account for the large number of mutants missing from our datasets (data not shown). This

suggested that if *tbp*$^{\Delta N/\Delta N}$ fetuses survived to birth, *tbp*$^{\Delta N/+}$;*rag*$^{-/-}$ mothers were rapidly consuming their mutant pups. Two possibilities could account for this behavior. First, it was possible that *tbp*$^{\Delta N/+}$;*rag*$^{-/-}$ females were selectively killing and consuming their mutant pups, or second, these females merely consumed that which was already dead. The prior scenario would imply that mutant pups are subject to infanticide. The latter scenario suggests mutant pups die on their own accord.

The *rag-1* knockout line is on an inbred C57Bl/6 background. These mice generally drop their litters during nighttime hours and, as they do so, consume the placentas and other extraembryonic tissues of their pups (personal observations). Generally, C57Bl/6 mice will partially or wholly consume any dead pups as well, which are commonly found in larger litters or first litters (personal observations). The C57Bl/6 females used in these experiments were fist-time mothers. It is also known that nulliparous C57BL/6 females have higher rates of infanticide than other mouse strains (Brown et al., 1999). To test whether *tbp*$^{\Delta N+}$;*rag*$^{-/-}$ females were selectively killing and consuming their mutant offspring, all pups taken by Cesarean section from these mothers just before natural birth on E18.5. All pups were fostered to nulliparous females of a different strain, CD1. CD1 females are an outbred mouse strain and are known to have excellent maternal behavior (personal observations). All CD1 foster mothers used had age-matched litters of their own. After fostering, the CD1 foster cages were checked for any dead pups. The results of these experiments demonstrated that while we fostered near Mendelian ratios of live mutant pups to CD1 mothers, none of the mutant pups survived to weaning (Table 4) and many of dead mutants were recovered, intact, from CD1 cages. In contrast, 90% of wild type pups did survive to weaning, indicating that mutant pups did not die because

Table 4. *tbp* Genotype Ratios of Pups Fostered to CD1 Mothers[a]

	$tbp^{+/+}$	$tbp^{\Delta N/+}$	$tbp^{\Delta N/\Delta N}$	n
# of pups Fostered	10	15	8	33
# of pups Weaned	9	8	0	17
% Survival of Pups	90%[b]	53%	0%	-

[a]All pups were from $tbp^{\Delta N/+}$;$rag^{-/-}$ females crossed to $tbp^{\Delta N/+}$;$rag^{+/+}$ males.
[b]Percent survival based on number of pups that survived to weaning versus how many pups were fostered.

of the fostering process. Additionally, as evidence of the exceptional maternal nature of CD1 females, it can be noted that during the fostering experiments one particular CD1 foster mother continued to keep dead mutant pups in her nest with live pups. These data suggest that although $tbp^{\Delta N/+}$;$rag^{-/-}$ females rapidly consume their mutant pups after birth, they do so because these pups are dead or dying. The fact that CD1 mothers do not eat dead mutant pups is likely due to differing maternal behavior between C57Bl/6 and CD1 strains.

$tbp^{\Delta N/\Delta N}$ Pups Survive Through Gestation in $tbp^{\Delta N/+}$;$rag^{-/-}$ Females

During our experiments to take litters by Cesarean from $tbp^{\Delta N+}$;$rag^{-/-}$ mothers above, we observed 69% of the expected number of $tbp^{\Delta N/\Delta N}$ pups at this point in gestation (Table 5). Mutant pups were runted (Fig. 9A) and when weighed, were found to be significantly lighter than their wild-type littermates (Fig. 9B). At Cesarean section, mutant pups were alive and responded to touch like their heterozygous and wild-type littermates. However, $tbp^{\Delta N/\Delta N}$ pups usually died shortly after Cesarean section. As described above, all mutants taken by Cesarean section and fostered to CD1 females were dead by the next day.

Table 5. Genotype Ratios at Cesarean Section

Maternal Genotype[a]	*tbp*$^{+/+}$	*tbp*$^{\Delta N/+}$	*tbp*$^{\Delta N/\Delta N}$	n (litters [ave. size])
tbp$^{\Delta N/+}$;*rag*$^{-/-}$	32 (100%)	56 (88%)	22 (69%)[b]	110 (15 [7.3])

[a]All females were crossed to *tbp*$^{\Delta N/+}$;*rag*$^{+/+}$ males.
[b]Percent survival based on expected Mendelian ratios and 100% survival of *tbp*$^{+/+}$ fetuses.

Differences Exist Between Wild Type and Mutant Placentas

At Cesarean section, we also collected all placentas from *tbp*$^{\Delta N+}$;*rag*$^{-/-}$ females. These placentas were stained using hematoxilin/eosin staining and we were able to distinguish all cell layers of the placentas (Fig. 10A). No significant differences were observed when wild type and mutant placentas were probed for the cell-specific genes *Pl1*, *Tpbp*, and *Gcm1* via *in situ* hybridization (data not shown). Using *in situ* hybridization, we survey several genes that are involved in normal placental function: *GATA-2*, *-3*, *LIF1r*, *TAP-1*,*-2*, *CD1.1*, *IRF-1*, and *p48*. We did not observe differential expression between wild type and mutant placentas for any of these genes (data not shown). However, as was the case for mutant pups, mutant placentas were significantly smaller in weight than their wild-type littermates (Fig. 10B).

For an additional and independent examination of mutant pups and placentas, we employed the expertise of an outside group of investigators to see if any defects could be detected. One litter of formalin-fixed, E18.5 fetuses and their placentas were sent to Dr. Phillipe LaBelle at the University of California at Davis. All specimens were sectioned and examined for pathologies that may contribute to the post-natal death of homozygous fetuses. No differences

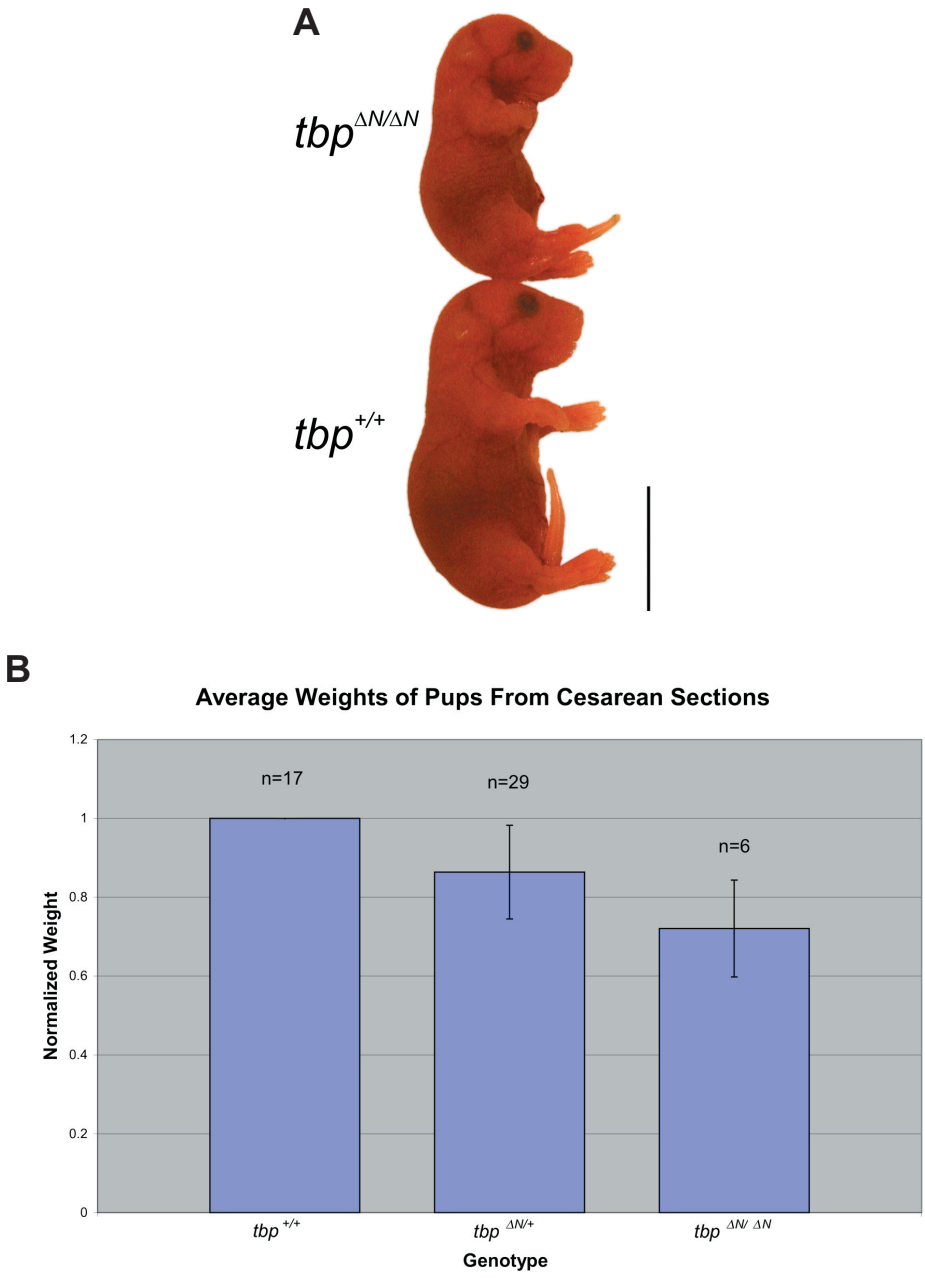

Figure 9. Mutant pups are approximately 70% the weight of their wild-type littermates at term (E18.5). A. Bar in photograph represents 1 cm. B. Pups were weighed and all genotype weights were normalized to the average weight of wild type pups within each litter. The bar charts were generated in the same fashion as described for Fig. 9 (wild-type standard deviation is 0.034). Mutant pup weights were significantly lower than those from the wild type pups of the same litter, $p<0.5$, based on student's t-test.

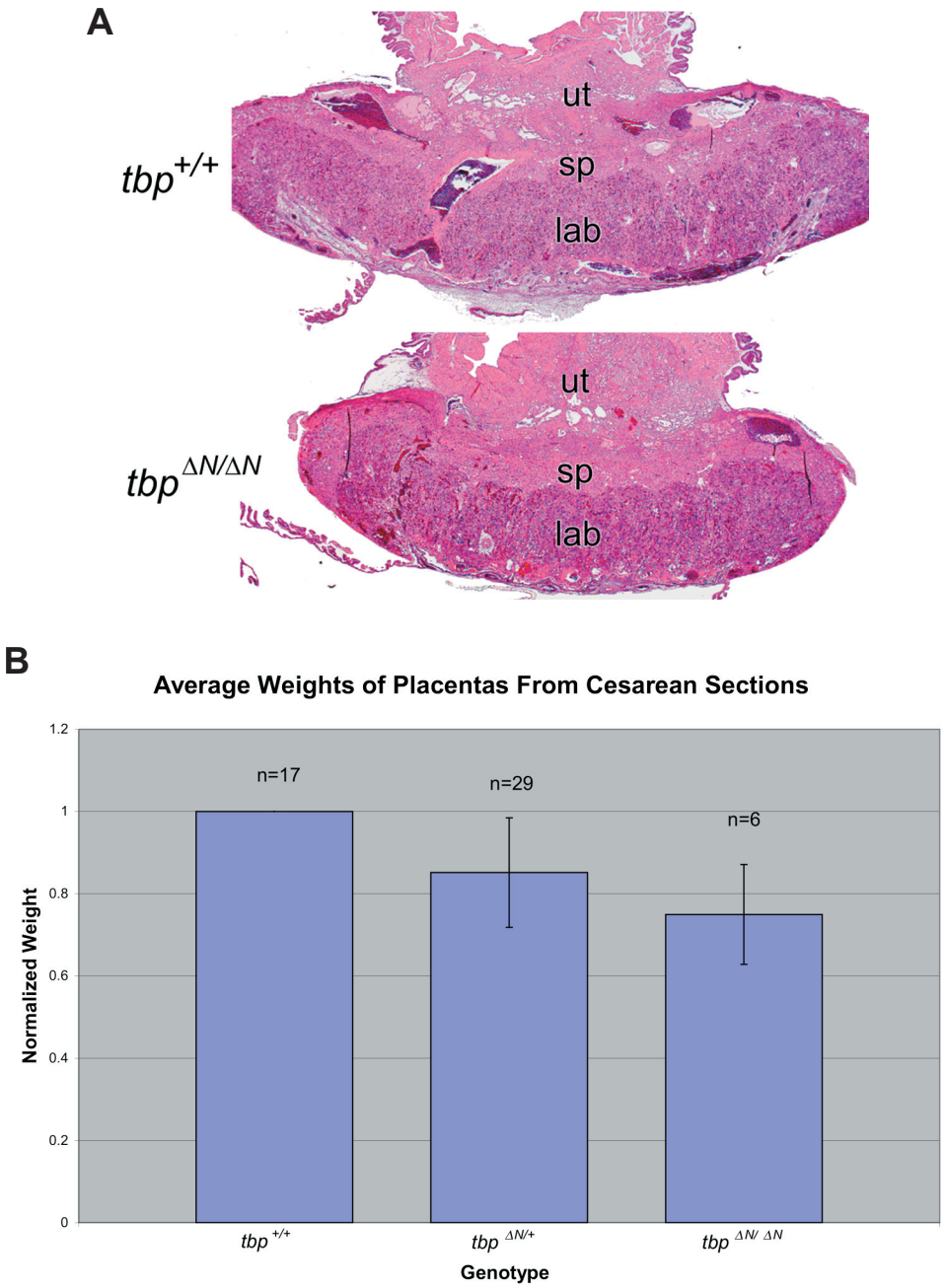

Figure 10. A. H/E stained wild type and mutant placentas from term (E18.5) litter. ut=uterus, sp=spongiotrophoblast, lab=labyrinth. B. Placentas were weighed and all genotype weights were normalized to the average weight of wild type placentas within each litter. The bar chart represents the averages of these normalized weights for each genotype. The standard deviation bar represents the deviations of the normalized weights (wild-type standard deviation is 0.037). Mutant placenta weights were significantly lower than those from wild type placentas of the same litter, $p<0.5$, based on student's t-test.

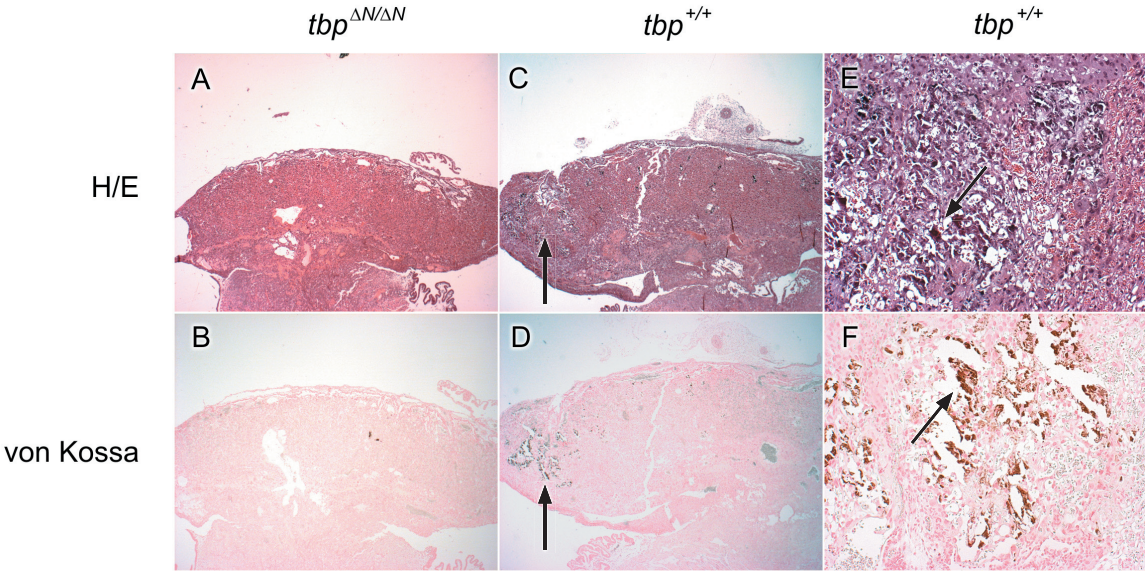

Figure 11. von Kossa and H/E staining of term (E18.5) placenta. A. H/E stained mutant placenta at (20X magnification). B. von Kossa stained mutant placenta (20X magnification). C. H/E stained wild-type placenta (20X magnification). D. von Kossa stained wild-type placenta (20X magnification). E. H/E stained wild-type placenta (100X magnification). F. von Kossa stained wild-type placenta (100X magnification). Note the decrease in brown precipitate in von Kossa stained mutant placentas (arrows).

between wild-type, heterozygous, or mutant fetuses were found in any tissues or organ systems in this litter. However, again differences between mutant and wild-type placentas were found. When placenta sections from this litter were stained using von Kossa stain, an agent which reacts with calcium phosphates and is usually used to detect calcium deposits in tissues, mutant placentas showed less staining in comparison to wild type placentas in this litter (Fig. 11). von Kossa staining in heterozygous placentas revealed an intermediate level of staining. The investigators responsible for these experiments reported these findings to be surprising, as changes in placental mineralization (calcification) is not a common finding. Nevertheless, together these observations suggest that differences exist between wild-type and mutant placentas and may be a clue to

why *tbp*$^{\Delta N/\Delta N}$ pups are runted and die post-natally.

Conclusion/Discussion:

Although most mutant fetuses survived past mid-gestation in immunocompromised *tbp*$^{\Delta N/+}$;*rag*$^{-/-}$ females, almost none survived to weaning (Table 2). Because C57Bl/6 mice have high rates of infanticide, especially as first time mothers (Brown et al., 1999), we fostered off pups from Cesarean sectioned *tbp*$^{\Delta N/+}$;*rag*$^{-/-}$ females to outbred CD1 females. Most wild type pups from the Cesarean sections survived when fostered to CD1 mothers, but mutant pups died (Table 4). Fostered mutant pups were found dead, but apparently uninjured in CD1 foster cages. Cesarean sections on E18.5 pregnant *tbp*$^{\Delta N/+}$;*rag*$^{-/-}$ females revealed that 69% of expected numbers of mutants survived through gestation (Table 5). Mutants responded to touch, albeit to a lesser degree than their wild-type and heterozygous littermates. Mutant pups and placentas were smaller than their wild-type littermates (Fig. 10A and B and 9A and B). Taken together, these observations suggested that mutant pups are not subject to infanticide by their C57Bl/6 mothers nor does fostering mutants to CD1 females increase their survival. We concluded that mutant pups that survive mid-gestation are likely born alive but compromised. Because of differences in placentas of mutants, we hypothesize that lethality of homozygous mutants is due to inherent defects, not to extrinsic factors like infanticide or birth-related complications such as premature detachment of the placenta from the uterus.

Removal of the N Terminus Results in Growth Restriction

Fetal development involves multiple complex events including the

differentiation of embryonic and extraembryonic lineages as well as intricate interactions with maternal tissues. Disruption of these events can lead to growth restriction and often this phenotype is secondary to the failure of the placenta to adequately supply nutrients to the fetus (Han and Carter, 2001). *tbp* mutant pups taken at E18.5 by Cesarean section are on average 70% the weight of wild type littermates (Fig. 9B). In weighing the placentas from these same litters, we found that mutant placentas, like the fetuses, were runted and were nearly 75% the weight of wild type placentas (Fig. 9A and B).

There are numerous studies that report intrauterine growth restriction (IUGR) as a result of mutations made in experimental mouse models. Commonly, these mutations disturb placental vasculature, compromising the placenta's ability to mediate the transport of nutrients, oxygen, and metabolic waste across the barrier dividing maternal and fetal compartments (Fischer et al., 2004). Mutations resulting in cell-specific defects of the placenta (Cross et al., 2002; Karaplis et al., 1994; Kingdom et al., 2000; Thumkeo et al., 2003) and disruption of endocrine functions (Cross et al., 2002) can also lead to growth restriction and fetal lethality. Many of the gene mutations affecting these placental functions are transcription factors. Disruption of the transcription factors Hand1 and Gcm1 affect aspects of placental development (Anson-Cartwright et al., 2000; Riley et al., 1998). Hand1 also functions by regulating the hormone placental lactogen I (Pl1) (Firulli et al., 1998; Riley et al., 1998). Our data suggest that although a placental defect was identified at midgestation, mutant placentas, albeit smaller, are sufficient to sustain the fetus through gestation in the absence of the adaptive immune system. Whether this decrease in weight of mutant placentas contributes to post-natal lethality remains to be determined.

Homozygous mutants for the *tbp*$^{\Delta N}$ mutation experience crises at midgestation and after birth. At midgestation, a placental defect was discovered and at the late crisis, placentas were runted. In removing the N-terminal portion of the protein, one could envisage disrupting interactions between this domain and other transcription factors involved in placental functions. Conversely, if the TBP N terminus functions to negatively regulate such factors, our mutation may result in activation of genes or pathways at inappropriate points in development. We observe a significant reduction in the size of homozygous pups (Fig. 9A and B) and their placentas (Fig. 10A and B) and conclude that the *tbp*$^{\Delta N}$ mutation leads to growth restriction and post-natal lethality in homozygotes, possibly because of diminished placental function.

The Late Crisis Does Not Involve an Adaptive Immune Response

The finding that mutant pups from *tbp*$^{\Delta N/+}$*;rag*$^{-/-}$ females died post-natally was surprising. Our data suggested that, unlike in the mid-gestational crisis, a maternal adaptive immune response was not involved in the late crisis. Although, it is possible that components of innate immunity could contribute to the late crisis, we have not observed any evidence of inflammation or monocyte infiltration in mutant placentas or fetuses (data not shown). While *rag-1*-null mice reportedly have increased numbers of NK cells (Mombaerts et al., 1992; Shinkai et al., 1992), the absence of mature T and B cells results in a decrease in NK cell activation (Carnaud et al., 1999). Thus, the *tbp*$^{\Delta N}$ mutation leads to postnatal lethality that does not likely involve an adaptive immune response.

The TBP N Terminus is Required Throughout Development

The N terminus of TBP appears to be required throughout development. We can rescue homozygous mutants for the *tbp*$^{\Delta N}$ mutation at midgestation by eliminating a maternal immune response. However, mutants pups still die postnatally, apparently independent of a maternal immune response. Thus, it is possible that the TBP N terminus is required differentially at these two crisis points. In the literature, there are numerous examples of genes that are essential for development in at different times and in different mechanisms. An example of such a gene is *Hand1*. Hand1 is a bHLH transctiption factor required in early gestation for extraembryonic functions, but is also required later for cardiac development (Firulli et al., 1998; Riley et al., 1998). *Hand1*-null embryos arrest early in development due to extraembryonic defects, but can be rescued by supplying *Hand1* mutants with wild type tetraploid placentas using diploid/tetraploid aggregations. Although the placental requirement for *Hand1* is met in these experiments, the mutants arrest at E10 due to a heart defect (Riley et al., 1998). Because we observe two separate and characteristically different points during gestation where we loose *tbp*$^{\Delta N/\Delta N}$ animals, it is possible that the N terminal domain of TBP may be similarly required for different mechanisms through development.

In contrast, it is also possible that the two crisis points where we loose most of the homozygous mutants are due to the same defect. For example, the placental defect that we observe at midgestation does not result in mutant fetal death, so long as the mother cannot mount an adaptive immune response. However, eliminating this response may only be postponing the inevitable death that mutants will face once required to live independently of the maternal

system. Given that mutant pups are runted at late gestation, it is possible that a single placental defect arises early in gestation, and compromises the growth of mutants throughout development, leading to a compromised pup at birth.

All results thus far suggest that a placental defect is responsible for the lethality we observe in homozygous mutants. *tbp*$^{\Delta N/\Delta N}$ placentas were smaller than their wild-type controls and staining with von Kossa indicated that there may be decreased calcium deposits in mutant placentas. We do not yet understand the mechanisms behind these differences and whether they play a role in the survival of *tbp*$^{\Delta N/\Delta N}$ pups. We also have not ruled out the possibility of concurrent defects in the fetus proper. Many functions that the placenta performs are dependent on signals directly from the fetus proper. Indeed, there are studies that confirm communication between placenta and fetus is critical and can even result in intrauterine growth retardation, if perterbed (Garnica and Chan, 1996). Thus, we must consider that death of *tbp*$^{\Delta N/\Delta N}$ pups may not be due solely to placental defects and that the interactions between placenta and fetus may play a role as well. Taken together, the data presented here suggest that beyond the requirement for a β2M-dependent placental function at midgestation, the TBP N terminus is required in pathways throughout development that are important for normal birth weight and survival of the neonate.

CHAPTER 5

THE USE OF CHIMERA GENERATION TO STUDY PLACENTAL DEFECTS

Introduction

Spontaneous duplication of the mammalian genome occurs in approximately 1% of all fertilizations. Although one or more genome duplication events are thought to have played a role in vertebrate evolution, polyploidy of mammals is usually incompatible with normal development and function in all but a few tissues (reviewed in Eakin and Behringer, 2003). Some exceptions do exist, however. For example, hepatocytes, myocardium, skeletal muscle, urinary bladder epithelia, syncytiotrophoblast, and corpora lutea can be polyploid due to endoreduplication or cell fusion during development (Keighren and West, 1993). In placenta, the trophoblast giant cells are considered to be polytene (having greater numbers of chromatids per chromosome) rather than polyploid (having greater numbers of segregating chromosomes) (reviewed in Eakin and Behringer, 2003). The molecular mechanisms that regulate the cells ability to be polyploidy or polytene, however, remain unclear (Eakin and Behringer, 2003).

In mice, development of tetraploid embryos has generally not been observed beyond midgestation (Tarkowski et al., 1977). However, tetraploidy of extraembryonic tissues in mice is more readily tolerated and the production of tetraploid:diploid (4N:2N) chimeras are widely used as a method for rescuing extraembryonic defects (Eakin and Behringer, 2003).

The most common method used for the production of tetraploid embryos is electrofusion by electrical stimulation (Senda et al., 1979). Two-celled embryos can be isolated and when a voltage difference across the plasma membranes of

the two blastomeres is generated, the closely apposed lipid membranes of the two cells are disrupted. This disruption creates transient cytoplasmic bridges between the two blastomeres, which expands until both cells are surrounded by one continuous plasma membrane. The result is a single celled embryo that is tetraploid. Initially, the blastomere of this embryo is binucleate diploid, but eventually, the two nuclei contribute chromosomes to a single tetraploid metaphase plate (Tarkowski et al., 1977).

When tetraploid embryos are aggregated with diploid embryos, the tetraploid cells tend to contribute more readily to extaembryonic tissues than to the fetus proper while diploid cells are restricted to the inner cell mass of the blastocyst that will become the embryo proper. The mechanism by which tetraploid cells are primarily restricted to the extraembryonic tissues is largely unknown but studies have shown that the fate of tetraploid cells is determined before implantation (~E7.5) (James et al., 1995). There are likely many factors that culminate in the lack of contribution of tetraploid cells to the embryo, including issues of fitness, compromised developmental potential, or differential rates of cell division. Another possibility that has been submitted to explain the uneven tetraploid cell distribution is that there could be a selective loss of tetraploid cells in the primitive ectoderm lineage, resulting in their absence in the fetus later in development (Eakin and Behringer, 2003).

Results

Chimera Generation to Address the Late Crisis

We considered that if the late crisis involved placental defects, we may

be able to rescue *tbp*$^{\Delta N/\Delta N}$ pups with the use of chimera generation experiments. Essentially, we wondered whether *tbp*$^{\Delta N/\Delta N}$ pups would survive the late crisis if they had developed on a *tbp*$^{+/+}$ placenta instead of a *tbp*$^{\Delta N/\Delta N}$ placenta. If *tbp*$^{\Delta N/\Delta N}$ pup survival increased as a result, we could conclude that the *tbp*$^{\Delta N/\Delta N}$ placenta plays a role in the late crisis. If, however, survival of *tbp*$^{\Delta N/\Delta N}$ pups did not increase as a result of being supplied a *tbp*$^{+/+}$ placenta, we could conclude that *tbp*$^{\Delta N/\Delta N}$ placentas are not the sole cause of the late crisis.

For these experiments, we utilized a BALB/c/C57Bl/6 hybrid strain of mouse generated by crossing BALB/c and C57Bl/6 mice. In this way, we could work with animals that were more robust than the pure C57Bl/6 mice while maintaining genetically identical backgrounds from individual to individual. To follow the development of tetraploid cells, we made use of a genetic marker, *8q* (described in Chapter 2). We generated a source of *tbp*$^{+/+}$ tetraploid cells by crossing Balb/c females with *tbp*$^{+/+}$ C57Bl/6 males that were homozygous for our *8q* marker gene. These matings resulted in BALB/c/C57Bl/6 hybrid zygotes that were *tbp*$^{+/+}$ and heterozygous for *8q* (Fig. 7). For a diploid source of cells, *tbp*$^{\Delta N/+}$; BALB/c/C57Bl/6 F1 or F2 hybrid females were crossed with *tbp*$^{\Delta N/+}$; BALB/c/C57Bl/6 F1 or F2 hybrid males. Pseudopregnant *tbp*$^{+/+}$; BALB/c/C57Bl/6 F1 females were used for surrogates. Thus, the tetraploid placentas and surrogate mothers in these experiments had identical genetic backgrounds. Fetuses in these experiments were an F2 generation BALB/c/C57Bl/6 hybrid or later. This means that unlike the placenta or surrogate mothers, the genetic backgrounds of siblings in any given litter are genetically dissimilar.

In order to control for these experiments, we have bred *tbp*$^{\Delta N/+}$; BALB/c/C57Bl/6 males with either F1 or F2 *tbp*$^{\Delta N/+}$;BALB/c/C57Bl/6 females and examined genotype ratios at midgestation, late gestation and at weaning (Table

Table 6. Fetal/Pup Survival in BALB/c/C57Bl/6 Hybrids

Survival Through Midgestation[a] Maternal X Paternal[b]	*tbp*$^{+/+}$	t*bp*$^{\Delta N/+}$	*tbp*$^{\Delta N/\Delta N}$	n
tbp$^{\Delta N/+}$ X *tbp*$^{\Delta N/+}$	54 (100%)	104 (96%)[c]	24 (44%)	196
Survival Through Gestation (Before Birth)[d]				
tbp$^{\Delta N/+}$ X *tbp*$^{\Delta N/+}$	47 (100%)	104 (110%)	24 (51%)	175
tbp$^{\Delta N/+}$ X *tbp*$^{\Delta N/\Delta N}$	-	67 (100%)[d]	34 (51%)	101
Survival To Weaning[e]				
tbp$^{\Delta N/+}$ X *tbp*$^{\Delta N/+}$	199 (100%)	374 (94%)	6 (3%)	579

[a]Fetal Harvests were performed at E14.5-E15.5.
[b]All Crosses involved BALB/c/C57Bl/6 Hybrid Parental Backgrounds.
[c]Percent survival based on expected Mendelian ratios and 100% survival of tbp$^{+/+}$ fetuses. [d]Percent survival based on expected Mendelian ratios and 100% survival of tbp$^{\Delta N/+}$ fetuses.
[d]Fetal Harvests were performed at E17.5-E18.5.
[e]Pup Genotypes were determined at approximately 3 weeks of age.

6). In hybrid females that were crossed to *tbp*$^{\Delta N/+}$; BALB/c/C57Bl/6 males, we observed 88% heterozygous and 44% homozygous mutant survival at mid-gestation. At late gestation, we observed 110% heterozygous and 51% homozygous survival. At weaning in hybrid females, 94% of heterozygous and 3% of homozygous mutant pups survived. These controls suggest that, if *tbp*$^{\Delta N/\Delta N}$ pups were to survive the late crisis when supplied with a tetraploid *tbp*$^{+/+}$ placenta, it is not likely due to F1 hybrid attributes of the placenta or mother. Rather, these pups would have survived to weaning because they developed on a *tbp*$^{+/+}$ placenta.

We generated chimeric embryos by aggregating 4N and 2N embryos together with a success rate of 50-60%, according to the PCR assay for the

Table 7. BALB/c/C57Bl/6 Hybrid Pup Survival on Wild-Type Tetraploid Placentas[a]

Maternal X Paternal			
tbp$^{\Delta N/+}$ X *tbp*$^{\Delta N/\Delta N}$	*tbp*$^{\Delta N/+}$	t*bp*$^{\Delta N/\Delta N}$	n
Survival At Cesarean Section	8	3	11
Survival To Weaning	7 (87.5%)[b]	0 (0%)	7 (64%)

[a]All pups were taken by Cesarean section on E18.5 and fostered to CD1 females. Total numbers taken by Cesarean section were 14 *tbp*$^{\Delta N/+}$ and 7 t*bp*$^{\Delta N/\Delta N}$ pups but table only represents those pups that were on *8q*$^{+}$ (tetraploid) placentas.
[b]Percentages of live pups at Cesarean section that survived to weaning. Pups were genotyped again at this time.

tetraploid cell marker, *8q*. However, we neither increased survival of *tbp*$^{\Delta N/\Delta N}$ pups (Table 7), nor achieved 100% tetraploid placentas by the current method used. The total number of pups obtained from these experiments, including those that were not on *8q*$^{+}$ placentas, was 21: 14 *tbp*$^{\Delta N/+}$ and 7 *tbp*$^{\Delta N/\Delta N}$ (52% of expected). This number of mutants at Cesarean section is comparable with the survival rates we found in control breedings (Table 6). Thus, the failure to increase survival of mutant pups in these experiments is likely due to the inability to replace defective *tbp*$^{\Delta N/\Delta N}$ placental cells with wild-type tetraploid cells. In order to understand why tetraploid contributions in the placenta are not 100%, it is pertinent to understand the lineage restrictions of the cellular compents of placenta during development and chimera production.

As described in detail in Chapter 1, three different cell types can be distinguished in the mouse blastocyst: trophectoderm, primitive endoderm, and primitive ectoderm. Each of these three lineages has different fates in the developing embryo and placenta (Fig. 12). Trophectoderm exclusively

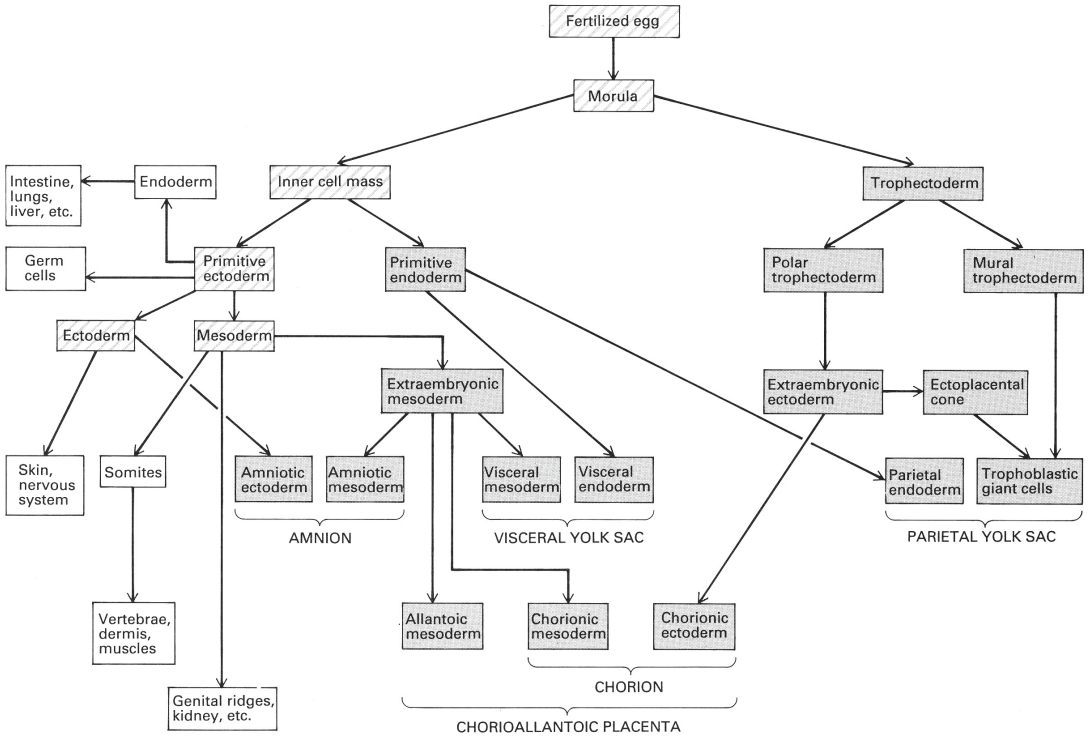

Figure 12. Fate map of the lineages of tissues constituting the mouse embryo and placenta. All tissues that will generate the embryo proper and extraembryonic cells are in hatched boxes. Tissues that will give rise to extraembryonic tissues are in gray boxes and tissues that will generate the embryo proper are in white boxes. Modified from Hogan, et. al., 1994.

differentiates into the trophoblast layer of the placenta. Primitive endoderm generates the parietal and visceral endoderm. Primitive ectoderm form yolk sac mesoderm, amnion, and the embryo proper (Hogan et al., 1994; Nagy and Rossant, 2001). When aggregated with diploid embryos, tetraploid embryos can contribute to the trophoblast and primitive endoderm lineage, but not to the primitive ectoderm derivatives of the embryo proper (Nagy et al., 1990; Tarkowski et al., 1977). This means that within the chorioallantoic placenta, most of the labyrinth and all blood vessels will be derived from diploid cells only (Hogan et al., 1994). Together, this literature demonstrates that the diploid:tetraploid

embryo aggregation method generates placentas that are comprised of both diploid and tetraploid cells.

Mid- vs. Late-Gestation Rescue using Chimera Generation

In Chapter 3, we reported rescuing mutant fetuses from the midgestation crisis through the use of chimera generation: *tbp*$^{\Delta N/\Delta N}$ fetuses supplied a *tbp*$^{+/+}$ placenta survived midgestation (Hobbs et al., 2002). The discrepancy in results at mid- and late gestation is not altogether surprising. Tetraploid cells reportedly have cell cycles that are approximately 2 hr slower than diploid cells (Koizumi and Fukuta, 1996). And although it has been reported that, with the use of this method, only up to 50% of extraembronic tissues are tetraploid (Tarkowski et al., 1977), through gestation, tetraploid cell contributions decrease (Goto et al., 2002; Nagy et al., 1990; Tarkowski et al., 1977). This is presumable to be due to an inability of tetraploid cells to compete with diploid cells. It may also indicate while diploid cells continue to divide through gestation, tetraploid cell division ceases. Consequently, if high levels of *tbp*$^{+/+}$ tetraploid cells are necessary in the placenta to increase survival of *tbp*$^{\Delta N/\Delta N}$ pups at the late crisis, it is likely that such a requirement was not met in our experiments, especially if the *tbp*$^{\Delta N}$ mutation creates a defect in placental tissues not derived from trophoblast.

Conclusions

Gene knockout studies to study the molecular mechanisms essential for processes during pregnancy in mice continue to rapidly identify genes that are essential for developmental processes and embryogenesis. From such studies, it can be concluded that placental pathologies can result from mutations in a

number of molecular mechanisms involved in the development or function of trophoblast, mesenchymal or vascular constituents.

The use of chimera generation can be an advantageous tool for dissecting embryonic and placental defects that result from experimental mutations and can greatly aid in deciphering developmental processes. However, due to the nature of the diploid:tetraploid aggregation method, it may not always be possible to address placental defects. Although the use of aggregation chimeras is generally more popular because it can generate chimeras more rapidly (Wood et al., 1993), there are two other approaches that can be used in the generation of chimeric embryos.

The other two methods to generate chimeras involve the use of ES cells: ES cells can be directly injected into a tetraploid blastocyst-stage embryo or ES cells can be aggregated with tetraploid embryos (Nagy et al., 1990). One difference between the diploid:tetraploid embryo aggregation method vs. those that use diploid ES cells is the extent to which the tetraploid cells contribute to extraembryonic tissues. In diploid:tetraploid morula aggregations, resulting extraembryonic tissues are only partially tetraploid while methods using ES cells generally result in extraembryonic tissues that are mostly tetraploid (reviewed in Eakin and Behringer, 2003). This is due to the limited ability of ES cells to colonize trophoblast (Beddington and Robertson, 1989). Thus, given our results and the available literature on diploid:tetraploid aggregation chimeras, testing for late-term placental defects may only be feasible using ES cell-associated methods.

CHAPTER 6

SUMMARY AND CONCLUSIONS

We have generated a mouse line that harbors a mutation in a component of the basal transcription machinery that has resulted in a tissue- or situation-specific defect. At midgestation, most *tbp*$^{\Delta N/\Delta N}$ fetuses die from a maternal immune response mounted against them, however, these fetuses can be rescued in three ways: 1) through the ablation of the maternal adaptive immune response; 2) through disruption of fetal β_2M-dependent processes; or 3) by supplying the mutant fetuses with a wild type placenta. A few *tbp*$^{\Delta N/\Delta N}$ fetuses do survive through midgestation and those that live to adulthood appear healthy and are fertile. Additionally, survival of *tbp*$^{\Delta N/\Delta N}$ fetuses past midgestation is not due to heritable traits, as *tbp*$^{\Delta N/\Delta N}$ animals do not have increased incidence of rearing *tbp*$^{\Delta N/\Delta N}$ pups. Rejection of *tbp*$^{\Delta N/\Delta N}$ fetuses is not likely elicited by a classical adaptive immune response because memory does not appear to be induced: rejection of *tbp*$^{\Delta N/\Delta N}$ fetuses in one pregnancy does not lead to a more rigorous rejection response in subsequent litters from the same female. These results suggest that, without the N terminus of TBP, inappropriate β_2M-dependent interactions occur between the fetus and mother, leading to a lethal adaptive immune response that eliminates most of the *tbp*$^{\Delta N/\Delta N}$ fetuses at midgestation.

In immunocompromised mothers, we found that *tbp*$^{\Delta N/\Delta N}$ fetuses survive through gestation but succumb to a second, late crisis. Before birth, homozygous fetuses are runted and when taken by Cesarean section, die shortly thereafter, even when fostered to CD1 females. We found that the placentas of homozygous mutants were also smaller in comparison to wild type littermate controls. When stained for calcium, mutant placentas were observed to have

less calcium deposits than wild type controls, which may be indicative of reduced nutrient transfer *in utero*.

The N terminus of TBP is shared by all vertebrates, including those that predate the appearance of placenta, and thus, likely functions in pathways that are more basal than those required for placenta. Although we did not detect defects outside of the placental defect in mice harboring the *tbp*$^{\Delta N}$ mutation, redundant mechanisms may exist to compensate for the loss of function of the TBP N terminus. From these studies, we have posited that the N terminus of TBP in mice participates in gene regulatory events in the placenta that ultimately protects the fetus from maternal immune rejection and is required for normal birth weight and survival of the neonate.

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APPENDICES

APPENDIX A

IN SITU HYBRIDIZATION AND SNAP FREEZING PLACENTAS FOR CRYOSECTIONING

1. Approximately 45 minutes before sacrificing pregnant female, pour 2-methyl butane into a 4 oz plastic urine cup with lid.
2. Place urine cup with lid on in a small Styrofoam cooler (that has a good lid) and pour CO_2 pellets around cup so that CO_2 pellets are packed around cup but so the lid of the cup is still accessible.
3. Allow 2-methyl butane to chill for ~30 minutes. Then, drop CO_2 pellets into 2-methyl butane and fill cup ~3/4 full of CO_2 pellets. Replace lid of cup and also top of cooler.
4. After removing placenta from uterus, place placenta on 1X PBS wet Whatman paper on ice (a good way to do this is to put ice in a Petri dish, close the lid and put wet Whatman paper on top of it).
5. With thin cryosection blade (normally for use in a cryostat), bisect placenta with one long sawing motion, using forcepts to keep the two halves in place.
6. Fill a cryomold (15mm X 15mm is best) with OCT medium and place placentas in OCT cut side down (try to be consistent with either having the embryo sides facing each other, or the uterus sides facing each other from section to section).
7. Bend edge of cryomold up so as to be able to grab edge with forceps. Place cryomold with placenta just at surface of 2-methyl butane so that the bottom freezes and holds placentas in the orientation you want.
8. Plunge cryomold down into 2-methyl butane and hold it against the submerged CO_2 pellets (where it is the coldest) for ~10 seconds. Let go of cryomold and replace top to 2-methyl butane container, leaving cryomold submerged until boiling has ceased and no bubbles are coming up (~30-45 seconds).
9. Keep frozen cryomolds on CO_2 pellets while working and store in airtight

baggie at -80^{o}C.

APPENDIX B

CESAREAN SECTIONS ON TERM PREGNANT MICE AND FOSTERING OF PUPS

1. On E18.5 of pregnancy (or late on E17.5, depending on size and behavior of mother), sacrifice pregnant female by cervical dislocation, taking caution to not put too much pressure on animal below shoulders.
2. Wet down fur with 70% ethanol and open body cavity. Remove entire pregnant uterus from body by cutting at each end by ovaries and the membranes along the uterus.
3. Place pregnant uterus on either waxed butcher paper or bench paper with plastic side up. Place a heating pad on “low” under waxed paper.
4. Starting at pup closest to the vaginal tract, begin cutting uterus open and, without touching fetus, open yolk and amniotic sacs with scissors. Use blunt-end forcepts for maneuvering. Cut umbilical cord close to pup, leaving approximately 2-3mm attached.
5. Immediately get pup out of amniotic fluids and blot mouth and nose with a kimwipe several times until pup begins arching head back and gasping.
6. Repeat for each pup, rotating and blotting all pups intermittently so they do not overheat lying on waxed paper. Do not let pups get too cold. Pups may be weighed and tail-snipped for genotyping at this time.
7. Obtain a CD1 female that has given birth within the last 24 hours and remove her from the cage with her litter. Keep her as far away from the cage with her pups in it as possible while introducing the new pups in with her litter.
8. If the total number of CD1 pups plus Cesarean sectioned pups is greater than 9-10, remove some of the CD1 pups from their cage and euthanize in a jar using isofluorane.
9. Place Cesarean sectioned pups in with remaining CD1 pups. Total number of pups should not exceed 9-10. Mix Cesarean pups with litter and feces

from CD1 mother and mix these pups in with CD1 pups.

10. Replace CD1 mother back in cage with Cesarean and CD1 pups and leave undisturbed overnight. Begin checking for any dead pups on the next day.

APPENDIX C

HARVEST AND TRANSFER OF EMBRYOS AT MULTIPLE STAGES

1. For all embryo harvests and transfers, females are induced to superovulate to obtain the greatest number of embryos per female (~20-30 embryos/female). This is accomplished by an intraperitoneal (IP) injection of 7.5IU pregnant mares serum (Calbiochem) at 1:30 p.m. (this time is based on a 7 a.m.-7 p.m. lights on and 7 p.m.-7 a.m. lights off cycle).
2. This is followed by an IP injection of 5 IU human chorionic gonadotropic (Calbiochem) 46 hours later to induce synchronized ovulation. At this time, females are set up 1:1 with desired studs. Additionally, females that are to serve as surrogates are set up with vasectomized females at this time as well. The next day, designated E0.5, all females are checked for the presence of a vaginal plug. Only those animals that have a plug should be used for experiments. However, the females that are to be used as surrogates can be re-set up with vasectomized males one more night if they did not plug the first night.
3. Whether embryos are to be harvested on E0.5 or E1.5, sacrifice those females that plugged on E0.5 (or E1.5) by cervical dislocation and remove oviducts by cutting across both the uterus and ovaries, taking care to not pull on oviducts and place in a dish of M2 media (Sigma). For E2.5 embryos, sacrifice E2.5 pregnant females by cervical dislocation and remove both oviducts AND entire uterus. Place E2.5 oviducts and uterus in M2 and separate by cutting through each uterine horn.
4. Observe under a dissecting microscrope. With watchmaker forcepts, locate the swelling of fertilized eggs in the oviducts for E0.5 pregnancies and tear oviduct to release eggs. For E1.5 pregnancies, locate infundibulum and insert a M2-containing 1cc syringe with an 181/2 gage needle that has been

cut blunt and polished. Push ~0.3ml of M2 though oviducts to flush out E1.5 embryos. For E2.5 embryos, flush BOTH oviducts and uteri with M2. For flushing uteri, insert same needle used to flush oviducts into the vaginal tract and flush one side of uterus at a time while pinching off other side with watchmaker forcepts. Collect eggs/embryos using a glass pipette attached to a mouth pipette apparatus and transfer to hyaluronidase/M2 for ~30 seconds to remove cumulus cells (skip this hyaluronidase/M2 step for E1.5 and E2.5 embryos and go directly to step 6).

5. Rinse eggs/embryos in M2 media and then rinse eggs/embryos again in M16 (Sigma).
6. Place eggs/embryos in a drop of M16 under a layer of light mineral oil and incubate at 37^{o}C/5% CO_2 until ready to use.
7. For E0.5, E1.5, and E2.5 embryo transfers, start by anesthetizing a pseudopregnant surrogate female with avertin. Avertin is injected IP and animal weight determines dose (wt (g) X 0.018). Use a 1cc or 0.5cc syringe fitted with an 181/2 gage needle to inject avertin.
8. Shave back of mouse using shears, being careful not to cut this skin. Sterilize skin surface by scrubbing with 70% ethanol-soaked kimwipes. Repeat scub 3 times.
9. Load ~10 embryos into each of two glass pipettes by first loading a small amount of M2, followed by a bubble of air, followed by embryos in M16, followed by another bubble, and followed finally by another small amount of M2.
10. Make incision in skin with sterilized scissors in the region of the back where an ovary is located. Make a small cut through the body wall and use scissors to tear an opening in the body wall that is large enough pull fat pad connected

to the ovary through. Locate fat pad and gently pull fat pad and ovary up through body wall and secure outside of the body using a clamp.

11. For E0.5 or E1.5 transfers, while observing oviducts under the dissecting microscope, tear open the Bursa covering the oviducts and locate the infundibulum using watchmaker forcepts. Insert the glass pipette containing embryos into the infundibulum and blow embryos into oviduct without blowing air into as well. For E2.5 embryo transfers, while observing under the dissection microscope, make a small hole in the uterus near the uterotubal junction with a sterile 181/2 needle. Insert glass pipette containing E2.5 embryos into hole and blow embryos into uterus without blowing air inside as well.
12. Gently move fat pad and ovary (and uterus, if applicable) back through body wall and into the body cavity. Seal incision in skin with surgical glue. Repeat steps 9-11 for the other side of the animal.
13. Place animal back in a cage that is situated on a heating pad set on “low”. Let animals recover from anesthesia on heating pad.