UNIVERSITY OF CALGARY

PLACENTAL CELL LINEAGE TRACING USING CRE RECOMBINASE TRANSGENIC MICE

by

Amanda Lea Fortier

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE

DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY

FACULTY OF MEDICINE

GRADUATE PROGRAM IN BIOCHEMISTRY AND MOLECULAR BIOLOGY

CALGARY, ALBERTA

JANUARY, 2005

UNIVERSITY OF
CALGARY

PLACENTAL CELL LINEAGE TRACING USING CRE RECOMBINASE TRANSGENIC MICE

Master of Science 2004

Department of Biochemistry and Biology, University of Calgary

ABSTRACT

The placenta is essential for the proper growth and development of the fetus. Cell lineage relationships in the placenta have not been studied *in vivo*, largely because cell lineage tracing methods were not easily adapted to study this organ. The use of site specific recombinases *in vivo* has provided feasible methods for the study of tissues that were previously unavailable for direct cell lineage studies, including the placenta. We have exploited the heritability of Cre recombinase mediated genetic changes to study the fate of cells in the placenta. We have generated mice in which Cre recombinase is under the control of the *Tpbpa* promoter, and examined the fate of cells expressing the transgene *in vivo*. *Tpbpa*-expressing cells were found to contribute to the secondary trophoblast giant cell lineage, and to be the predominant cell type associated with maternal spiral arteries and large maternal blood spaces within the labyrinth layer.

ACKNOWLEDGEMENTS

First, I would like to thank my supervisor, Jay Cross, for all the time and effort he has spent helping me to develop critical thinking skills. Jay has been encouraging through the difficult times. He has always tried to get me to see things in a different way, and to approach things from a different angle, an invaluable lesson for the future.

I would like to thank all of the members of the Cross lab that I have had the pleasure of working with over the past few years. In particular, I want to thank Erica Watson, Colleen Geary-Joo and Maja Starovic, who have all taught me invaluable lessons in both science and in life. The wonderful postdocs who have passed through the lab (Hideyuki Yamamoto, Myriam Hemberger, David Simmons, David Natale, Micheal Hesse and Haruo Nakano) have been a constant source of so many things – knowledge, assistance and encouragement. Of course, no mention of the Cross lab would be complete without Fran Allen, Martha Hughes, Lin Su, and Xiang Zhao who are the mainstays of the lab, and always willing to help whenever they are needed.

I would like to thank all the many ultimate players I have had the joy and happiness to play with here in Calgary, for making my life so much more fun!

Finally, and most importantly I want to thank my family and friends. My parents and siblings have been so patient in my pursuit of higher learning; despite how far from home it is taking me. Jeremy Hebert, you have been my source of strength – I love you and your family. Although our time together is short, our friendship is forever.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AP	alkaline phosphatase
bp	base pairs
BSA	bovine serum albumin
diI	1,1’-dioctadecyl-3,3,3’,3’-tetramethyl indocarbocyanine perchlorate
diO	3,3 ′-dioctadecyloxacarbo-cyanine
DNA	deoxyribonucleic acid
E	embryonic day
EGFP	enhanced green fluorescent protein
EPC	ectoplacental cone
ES	embryonic stem
FRT	Flp recombinase recognition target
hPLAP	human placental alkaline phosphatase
HRP	horseradish peroxidase
ICM	inner cell mass
IRES	internal ribosome entry site
JG	juxtaglomerular
loxP	location of crossover (x) in P1 bacteriophage
PCR	polymerase chain reaction
RDX	rhodamine-conjugated dextran
TE	trophectoderm
tet	tetracycline
tetO	tetracyline operator
Tpbpa	trophoblast specific protein alpha

CHAPTER ONE:

INTRODUCTION

1.1 General Introduction

The establishment of a mature placenta is critical for the growth and development of the fetus. Defects in placental development have been associated with many problems of pregnancy, including growth restriction, preeclampsia and death. Understanding how the placenta develops will assist in the comprehension of the problems that arise during pregnancy, and improve the chance for successful outcomes.

In the mouse, the mature placenta contains three main trophoblast cell types; the hormone-producing trophoblast giant cells, the supportive spongiotrophoblast layer and the syncytiotrophoblast separating maternal and fetal blood (Cross et al., 1994) (Figure 1). These cell types all arise from the same cell type, the trophectoderm (Cross, 2000), but after the initial formation of this progenitor population, little is known about the cell lineage decisions that lead to the generation of the three distinct cell types in the mature placenta. Indirect evidence, largely through cell and explant culture experiments, suggests that there are two stages of differentiation. The first decision establishes the extraembryonic ectoderm and the ectoplacental cone, and the second establishes the final phenotype of the cells (Figure 2).

Direct cell lineage tracing experiments are important to the understanding of the molecular mechanisms underlying trophoblast differentiation, but in the mouse placenta have been difficult as this tissue is not readily manipulated *in vivo*. As new techniques have been developed for studying cell lineage *in vivo*, the ability to introduce permanent, heritable genetic change using site-specific recombinases has made cell lineage tracing in the placenta possible. We have exploited the Cre-loxP system to study cell lineage in the placenta. This introduction will focus on our current understanding of placental development and the lineage decisions that lead to the generation of the terminal cell types, cell lineage tracing methods and applications of site-specific recombinases in general.

1.2 Placental Development

1.2.1 Early Development of the Trophoblast Lineage

The trophoblast lineage is the first cell type to be specified in the developing mouse embryo. It first appears at the blastocyst stage as a simple epithelium termed the trophectoderm (TE) (Figure 1) (Cross, 2000). These cells surround the outside of the blastocyst, enclosing the inner cell mass (ICM), which will form the embryo proper, and the blastocoel. At this early stage, only 50-60 trophoblast cells are formed, such that a significant amount of proliferation occurs following implantation (Cross, 2000). The signal for proliferation originates in the ICM, so placental development depends on the presence of a viable embryo (Cross, 2000; Rossant and Tamura-Lis, 1981). The proliferative population of trophoblast cells is limited to the cells of the polar TE, which are immediately overlying the ICM (Figure 1) and later to the extraembryonic ectoderm of the chorion (Cross, 2000). The mural TE cells, which are the cells surrounding the blastocoel, cease proliferating and terminally differentiate to form primary trophoblast giant cells immediately following implantation (Cross, 2000).

1.2.2 Cell Types in the Mature Placenta

Within the mature placenta, there are three main layers of trophoblast cells: the outermost layer of trophoblast giant cells, the intermediate spongiotrophoblast layer and the labyrinth closest to the embryo (Figure 1) (Cross et al., 1994). The labyrinth forms after embryonic day (E) 9 following chorioallantoic attachment. The labyrinth is mostly made up of two layers of trophoblast syncytia separating the maternal blood spaces from the fetal blood vessels (Adamson et al., 2002; Cross, 2000). The spongiotrophoblast layer is derived from a flattening of the ectoplacental cone after E7.5 (Cross, 2000). Trophoblast giant cells lie at the periphery of the placenta and first mediate invasion and implantation into the uterus (Cross et al., 1994). Following implantation, trophoblast giant cells interact with the maternal blood vessels, and produce hormones, growth factors and angiogenic factors that affect maternal physiology (Adamson, et al. 2002; Cross et al., 1994; Hemberger et al., 2003b).

Figure 1. Development of extraembryonic cell lineages. Development of the mouse embryo from embryonic day (E) 3.5 to 12.5, highlighting the development of extraembryonic lineages. ICM=inner cell mass. Adapted from Rossant and Cross (2001).

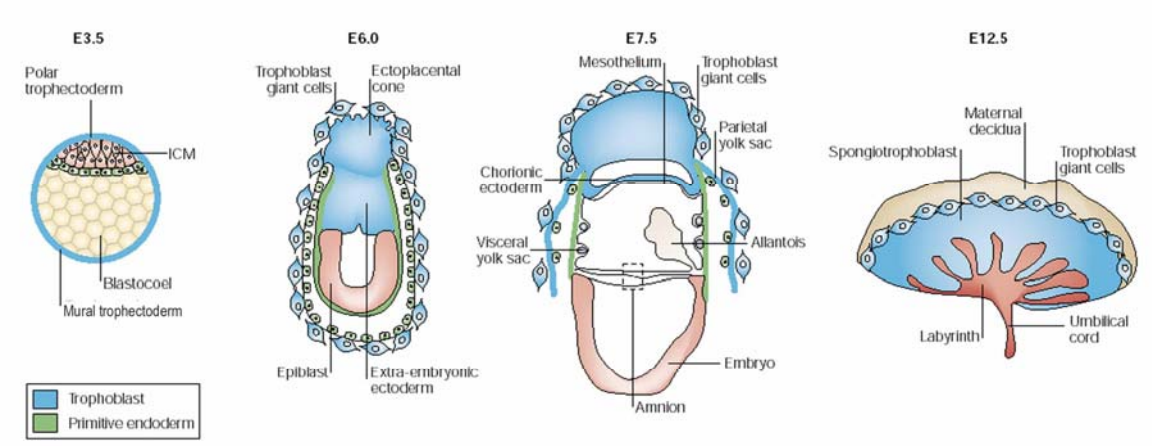


Figure 1. Development of extraembryonic cell lineages. Development of the mouse embryo from embryonic day (E) 3.5 to 12.5, highlighting development of the extraembryonic lineages. ICM= inner cell mass. Adapted from Rossant and Cross (2001).

1.2.2.1 Labyrinth

The development of the labyrinth is dependant upon the interaction of the allantoic mesoderm with the chorionic trophoblast cells (Cross, 2000). The allantois grows out from the posterior end of the embryo and contacts the chorion at E8.5 (Downs, 1998). Within the chorion, some cells continue to proliferate, while some of the trophoblast cells begin to form primary branch points for the villous structure (Cross, 2000). Within these branch points, allantoic mesoderm and fetal blood vessels bud into the chorionic plate, these continue to elongate and branch to form the fetal blood spaces within the labyrinth.

Syncytiotrophoblast cell differentiation also begins at E9.0. (Adamson et al., 2002; Cross, 2000). Syncytiotrophoblast cells form by the fusion of precursor cells to form multinucleate cells (Cross, 2000). The precursor of this cell population has yet to be identified, but likely resides in the chorion based on the conserved expression of certain genes exclusively in the chorion and later in the labyrinth layer of the placenta (Cross, 2000). The bulk of the labyrinth is made up of the two layers of these syncytial cells, which surround the fetal blood spaces within the labyrinth (Adamson et al., 2002).

The factors required for trophoblast fusion in the mouse placenta are unknown. However, two genes involved in fusion of the analogous human villous cytotrophoblast cells have been identified, *syncytin-1*(Mi et al., 2000) and *syncytin-2* (Blaise et al., 2003). Of the two, *syncytin-1* has been shown to be expressed exclusively in the syncytial cells of the human placenta. The syncytin proteins are encoded by an envelope gene of the human endogenous retroviral family, and mediate cell fusion *in vitro* (Blaise et al., 2003; Chang et al., 2004). These genes are not present in mice, however, indicating that other mechanisms of cell fusion are likely responsible for fusion in other mammals.

1.2.2.2 Spongiotrophoblast and Glycogen Trophoblast

The spongiotrophoblast layer lies between the innermost labyrinth layer and the outer trophoblast giant cell layer, and is comprised of a densely packed layer of diploid cells (Cross et al., 2002a). The spongiotrophoblast layer is likely derived from the diploid precursor population within the EPC based on the conservation of gene expression patterns. Both trophoblast-specific protein alpha (*Tpbpa*) (Carney et al., 1993;

Lescisin et al., 1988) and FMS-like tyrosine kinase-1 (*Flt 1*) (He et al., 1999) are expressed in a subset of cells within the EPC, and become restricted to the spongiotrophoblast layer as placental development proceeds (Cross et al., 2003).

The function of the spongiotrophoblast layer is twofold. First, it provides structural support to the underlying labyrinth (Cross, 2000; Rossant and Cross, 2001). Evidence for this is provided by analysis of several genetic mutants with defects in spongiotrophoblast formation. In mice deficient for the basic helix-loop-helix transcription factor *Mash2*, the spongiotrophoblast layer is lost, with an associated increase in trophoblast giant cell formation (Guillemot et al., 1994). Additionally, these mice demonstrate defects in labyrinth morphogenesis, which was shown to be secondary to the spongiotrophoblast defect in chimaeric studies (Tanaka et al., 1997). Similarly, in the absence of *ARNT*, a subunit of hypoxia inducible factor-1 (*HIF-1)*, spongiotrophoblast maintenance is impaired and again, the trophoblast giant cell population is expanded (Adelman et al., 2000; Kozak et al., 1997). These mice also demonstrate defects in labyrinth morphogenesis, including decreased fetal blood vessel invasion. Chimaeric mice demonstrated that *Arnt-/-* mesodermal cells were able to invade into the placenta in a wild type context, indicating that the defect resides in the trophoblast compartment, though the experiment does not distinguish between roles in the spongiotrophoblast versus the labyrinth trophoblast cells. In a different context, *Cited1*-deficient mice demonstrate an expanded spongiotrophoblast layer with larger projections into the labyrinth layer, as well as defects in labyrinth morphogenesis characterized by larger, less branched maternal blood spaces (Rodriguez et al., 2004). Rodriguez *et al.* (2004) propose that the increase in the spongiotrophoblast layer may inhibit branching morphogenesis in the labyrinth by either physical or molecular means. Together, these data support the hypothesis that a major function of the spongiotrophoblast layer is to support the underlying labyrinth.

A second important role of the spongiotrophoblast is the production of hormones that are targeted to both maternal and fetal tissues (Lu et al., 1994). In particular, the spongiotrophoblast produces and releases proteins of the prolactin family of hormones and cytokines that are important for the establishment and maintenance of pregnancy

(Soares, 2004; Soares et al., 1998). This function is shared with glycogen trophoblast cells and trophoblast giant cells, but each cell type expresses distinct members of the prolactin family (Soares, 2004), suggesting distinct roles for each of these cell types in physiological contol.

Glycogen trophoblast cells first appear within the spongiotrophoblast layer, and express the spongiotrophoblast marker *Tpbpa* (Adamson et al., 2002). These cells likely represent a specialized subtype of spongiotrophoblast (Cross et al., 2003), and are known to invade into the maternal decidua by an interstitial route (Adamson et al., 2002), and secrete hormones important for the maintenance of pregnancy (Soares, 2004; Soares et al., 1998). These data suggest that glycogen trophoblast cells may play an important role in influencing maternal physiology for maintaining pregnancy and perhaps in preparing the maternal system for postpartum requirements such as lactation.

1.2.2.3 Trophoblast Giant Cells

Trophoblast giant cells are so named because of the large cell and nuclear sizes they achieve. Trophoblast giant cells exhibit an unusual form of DNA replication, in which the cells cease dividing but continue to replicate their DNA, reaching ploidies up to 1024C (Zybina and Zybina, 1996). In rodents, trophoblast giant cells arise in two distinct stages during placental development. The first trophoblast giant cells to differentiate (‘primary’ giant cells) result from the differentiation of the mural TE after implantation (Cross, 1998). A second set of trophoblast giant cells (‘secondary’ giant cells) is derived from a precursor cell population at the outer edge of the ectoplacental cone, and later from the spongiotrophoblast. The evidence for this developmental pathway is indirect, and lineage tracing studies have yet to be done. However, the isolation and culture of ectoplacental cone or spongiotrophoblast cells results in a rapid arrest of proliferation and differentiation to trophoblast giant cells (Carney et al., 1993). Trophoblast stem cells have been shown to reside in the chorion layer (Tanaka et al., 1998), and if these cells are cultured in the absence of growth factors they differentiate to form trophoblast giant cells after passing through an intermediate stage where the cells express ectoplacental cone markers (Carney et al., 1993). These data suggest that

trophoblast giant cells differentiate from an intermediate precursor population in the ectoplacental cone.

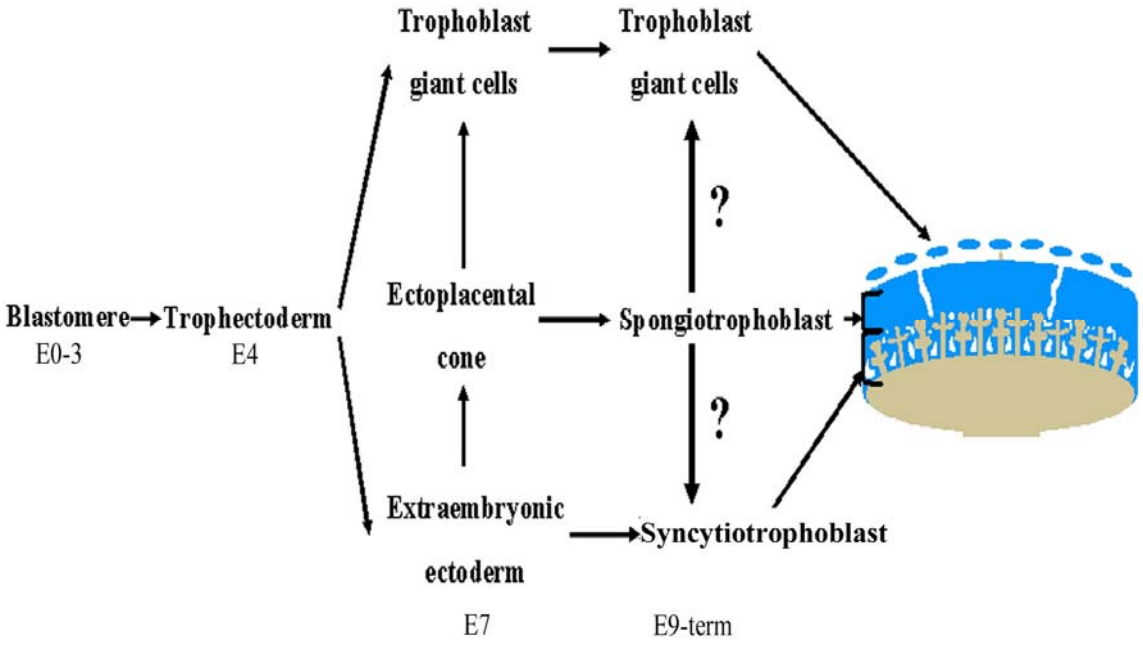
1.2.3 Trophoblast Stem Cells

Early studies of explant cultures showed that if extraembryonic ectoderm cells were cultured in close association with embryonic tissue, they continued to proliferate and some did not initiate differentiation to form trophoblast giant cells (Rossant and Tamura-Lis, 1981). In constrast, EPC cells in culture initiated giant cell differentiation regardless of culture conditions. Examination of known markers of placental cell subtypes in various explant cultures provided some clues as to cell lineage relationships (Figure 2) (Carney et al., 1993). When EPC cells are dissected and placed into culture, they express the spongiotrophoblast specific gene *Tpbpa* within 2 days, and then activate expression of the trophoblast giant cell specific genes *Pl1*, *Pl2* and *Plf* after 4 days (Carney et al., 1993). Cultured extraembryonic ectoderm cells also turn on these gene, though the sequence is delayed by about 2 days (Carney et al., 1993). These results suggest that cells of the extraembryonic ectoderm are capable of forming EPC cells, and that from EPC cells trophoblast giant cells can develop (Carney et al., 1993). These two sets of results suggest that extraembryonic ectoderm cells may serve as a stem cell population for the other trophoblast cell types (Rossant and Tamura-Lis, 1981). This suggestion has been validated by the isolation of trophoblast stem cells from the extraembryonic ectoderm of E6.5 to E8.5 conceptuses in the presence of fibroblast growth factor 4 (FGF4) (Tanaka et al., 1998; Uy et al., 2002).

1.3 Placental Vascularization

The establishment of maternal blood flow to the implantation site is vital to the successful growth and development of the fetus. The exchange of oxygen, nutrients and waste occurs within the labyrinth layer of the placenta, where maternal blood and fetal blood are brought into close proximity to facilitate the exchange.

Figure 2. Cell lineage relationships in the extraembryonic tissues of the mouse. Cell lineage relationships of trophoblast cells in the mouse based on experimental evidence. Direct cell lineage tracing has yet to be done in the mouse. E= embryonic day. (Adapted from Cross, 2000).


Trophoblast
giant cells
Trophoblast
giant cells
?
Blastomere
E0-3
Trophectoderm
E4
Ectoplacental
cone
Spongiotrophoblast
?
Extraembryonic
ectoderm
Syncytiotrophoblast
E7
E9-term

1.3.1 Maternal Vascular System

Maternal blood vessels grow towards each implantation site by angiogenesis, ultimately allowing blood to reach the developing placenta (Cross et al., 2002b). Maternal arteries enter through the maternal decidua, branching into several smaller arteries with a characteristic spiral shape (Adamson et al., 2002). These spiral arteries come together at the trophoblast giant cell layer to form large central canals. A range from 1 to 4 central canals has been observed in the mouse placenta. These central canals travel toward the fetal side of the placenta, where blood enters into the small blood spaces within the labyrinth. The maternal blood then drains into venous channels and exits the placenta where it returns to maternal circulation through wide veins (Adamson et al., 2002). This arrangement allows the most oxygenated blood to flow immediately to the fetal side of the placenta, and then flow back toward the maternal side as exchange of nutrients for waste occurs within the labyrinth.

Interestingly, maternal blood spaces within the labyrinth are not lined by endothelial cells, but rather by trophoblast cells (Adamson et al., 2002; Hemberger et al., 2003; Wooding, 1994). This type of placentation is referred to as 'hemochorial' (Wooding, 1994). Within the labyrinth, maternal blood spaces are surrounded by three layers of trophoblast cells; a layer of small cuboidal cells directly in contact with the maternal blood surrounded by two layers of syncytiotrophoblast cells (Adamson et al., 2002).

The maternal spiral arteries entering the placenta are also associated with trophoblast cells. These trophoblast cells invade into the maternal decidua by a peri/endovascular route, and replace the vascular endothelial lining (Adamson et al., 2002; Hemberger et al., 2003). This replacement is observed up to 300 μm away from the main trophoblast giant cell layer (Hemberger et al., 2003), and these cells express the trophoblast giant cell marker *Plf* (Adamson et al., 2002; Hemberger et al., 2003). Interestingly, these cells do not express another marker of trophoblast giant cells, *Pl1* (placental lactogen-I), suggesting that they may represent a specialized type of trophoblast giant cell (Cross et al., 2002b; Hemberger et al., 2003).

1.3.2 Fetal Vascular System

Fetal blood enters the placenta through a single umbilical artery. Fetal blood vessels grow into the labyrinth region through a process of concerted branching morphogenesis of the trophoblast cells, consisting of syncytiotrophoblast, trophoblast giant cells and mononuclear cells, and mesoderm, consisting of blood vessels and stromal cells (Cross et al., 2002b). In the region where it contacts the placenta, the single umbilical artery branches extensively, and the resulting arterioles grow up into the labyrinth (Adamson et al., 2002). Once the fetal arterioles reach the spongiotrophoblast layer, they become highly branched as they travel back toward the fetal side of the placenta. Fetal blood drains through venules into a single umbilical vein (Adamson et al., 2002). The arrangement of fetal arteries results in blood flow that runs countercurrent to the maternal blood, allowing for efficient exchange between the two blood compartments. In contrast to the maternal blood spaces, the fetal blood vessels are lined by endothelial cells throughout development (Adamson et al., 2002).

Although the morphological development of the mouse placenta is beginning to be understood, the molecular mechanisms underlying this development as well as the cell lineage decisions that lead to the development of three different trophoblast cell types remain unclear. The development of new methods for genetic analysis in mice, such as the application of site-specific recombinases will allow for the study of these early molecular mechanisms and cell fate decisions.

1.4 Site-Specific Recombinases

The development of site-specific recombinases has revolutionized genetic analysis in mice. These recombinases recognize defined target sites and catalyze recombination between the sites, resulting in genetic change. Several of these recombinase enzymes have been shown to be active in mammalian systems.

1.4.1 Cre-loxP System

Cre recombinase is a 38 kDa protein from the P1 bacteriophage that catalyzes recombination between two defined consensus 34 bp sites, called loxP (location of crossover (x) in P1 bacteriophage) sites (Hoess et al., 1984; Hoess et al., 1982). The loxP site consists of two inverted repeats of 13bp palindromic sequences, separated by an 8bp spacer (Figure 3) (Hoess and Abremski, 1984). Cre recombinase is a member of the λ integrase family of site specific recombinases. Members of the λ integrase superfamily share a common mechanism of DNA recombination involving strand cleavage, exchange and ligation (Branda and Dymecki, 2004; Sadowski, 1995). Two target sites are required for recombination to occur. These target sites are bound by recombinase monomers which promote the formation of a DNA synapse. Recombination then occurs within the spacers (Amin et al., 1991). The product of the recombination reaction is dependent on the orientation of the loxP sites (Figure 3). When the two loxP sites are oriented in a head-to-tail manner on the same chromosome, the recombination reaction is essentially irreversible due to the loss of a circular reaction product.

One attractive feature of this system is that Cre recombinase catalyzes this recombination in any DNA in any cellular environment (Nagy, 2000; Sauer and Henderson, 1988). This includes both actively dividing and post-mitotic cells, as well as in most tissue types (Branda and Dymecki, 2004).

1.4.2 Other Site-Specific Recombinases

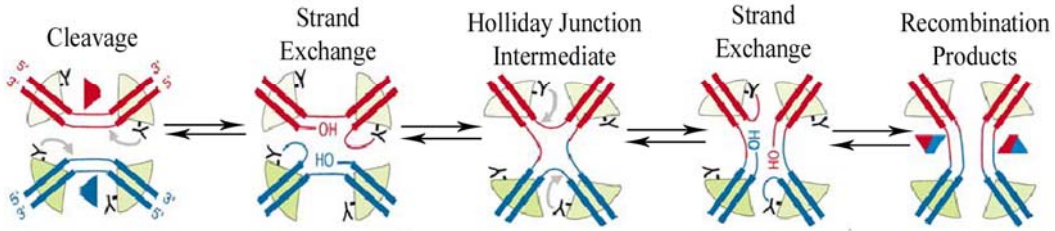
Another member of the λ integrase family, Flp, so named because of its' ability to "flip" DNA sequences in *Sacchromyces cerevisiae*, uses the same recombination mechanism as Cre recombinase. Flp catalyzes recombination between FRT (Flp recombinase recognition target) sites (McLeod et al., 1986), which share the same overall organization as loxP sites. The mechanism of recombination is the same as for Cre recombinase (Amin et al., 1991).

Figure 3. Mechanism of Cre recombination. (A) The sequence of a loxP site, consisting of two inverted palindromic repeats (black) and an 8 bp spacer (red). The black arrows indicate the two inverted palindromes. (B) Schematic representation of the model for Cre recombination. Four Cre molecules form a tetramer resulting in synapsis. DNA cleavage occurs at tyrosine residues, and nucleophilic attack of the released 5' OH groups results in Holliday junction formation. A second round of cleavage and strand exchange gives rise to recombination products. (C) Schematic representation of conditional gene activation. A stop sequence is flanked by two loxP sites in a head to tail orientation. In the presence of Cre recombinase, excision occurs releasing a circular product and allowing expression of the gene of interest. (Modified from Troche et al. 2002).

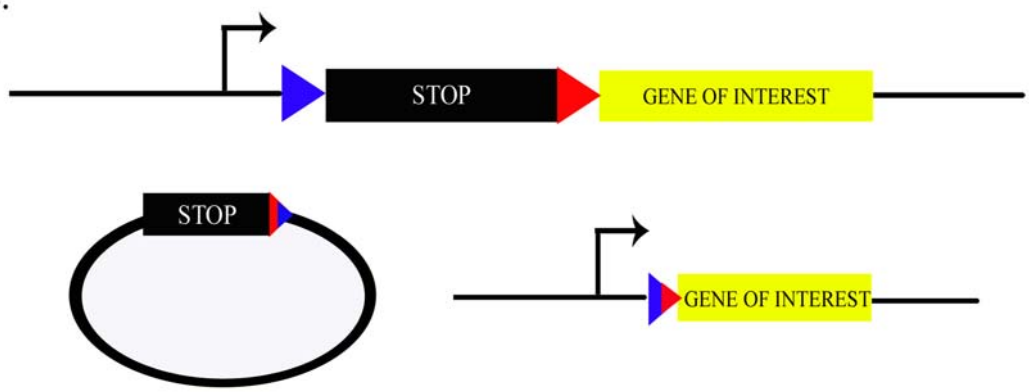
A.

5' ATAACTTCGTATA ATGTATGCTATACGAAGTTAT 3'
3' TATTGAAGCATATTACATAC GATATGCTTCAATA 5'

B.



C.



Another site-specific recombinase has recently been established for use in ES cells (Belteki et al., 2003). The ϕC31 site-specific recombinase is derived from *Streptomyces* and catalyzes recombination between two heterotypic recognition sites; attB is 34 bp long while attP is 39 bp long (Groth et al., 2000). These two sites contain imperfect inverted repeats and are thought to be bound by recombinase monomers (Groth et al., 2000). Due to the heterotypic nature of the recognition sites, the product sites are unable to catalyze further ϕC31-mediated recombination reactions (Belteki et al., 2003). Of significant importance is the finding that expression of ϕC31 does not inhibit the ability of ES cells to populate the germline when used to generate chimaeric mice (Belteki et al., 2003).

1.4.3 Conditional Genome Alteration in the Mouse

One very attractive feature of most site-specific recombinases is that they are active in both actively dividing and post-mitotic cell populations, and can catalyze recombination in most tissue types (Branda and Dymecki, 2004). This has allowed for the generation of a system for conditional genome alteration in the mouse, whereby a site-specific recombinase can be expressed in a spatially- and/or temporally-controlled manner to induce a genetic change only in the place or at the time desired. This can be accomplished by placing the expression of Cre recombinase under the control of a specific promoter, in order to express it only when and where the genome alteration is desired. The list of tissue-specific Cre recombinase bearing mice is increasing at a rapid pace, and already includes mice expressing Cre recombinase in most of the major organs and tissue types including the central nervous system (Cinato et al., 2001; Dragatsis and Zeitlin, 2000) primordial germ cells (Lomeli et al., 2000), male germ line (O'Gorman et al., 1997), female germline (Lan et al., 2004); endothelial cells (Gustafsson et al., 2001; Kisanuki et al., 2001) and heart (Agah et al., 1997; Sohal et al., 2001; Stanley et al., 2002). As of yet, no trophoblast cell type specific Cre mice have been described.

Using the Cre-lox system, a conditionally activated or inactivated transgene is possible. In order to conditionally activate or inactivate a gene, a construct is created in which expression is under the control of a ubiquitous promoter. The construct itself should contain two loxP sites flanking a DNA sequence, which can be a reporter gene,

such as a gene whose product can be assayed colorimetrically or a drug resistance gene or an extensive stop sequence. Alternately, if the goal is to conditionally inactivate a gene, this gene should be placed between the two loxP sites. If you wish to conditionally activate a gene, this gene is placed after the second loxP site (Figure 3). In the presence of Cre recombinase, the DNA between the two loxP sites is excised, resulting in activation or inactivation of the gene in question. This binary transgenic approach has been very useful in studying genes in which traditional knockout studies result in an embryonic lethal phenotype, precluding study of the gene function at later developmental stages. The growing number of tissue-specific Cre mice is leading to the ability to conditionally activate or inactivate genes in most tissue types without consequence to other tissues in the animal.

Several alternatives have also been developed to allow temporal control of Cre expression. Expression of Cre has been placed under the control of tetracycline (tet)-dependent gene regulatory systems (Gossen and Bujard, 1992). This system involves the expression of a transactivator gene that specifically binds tetracycline or its analog doxycycline, and the tet operator (tetO) sequence, resulting in transcription. The tetO is fused to the recombinase gene, such that the recombinase is only expressed upon transactivation (St-Onge et al., 1996). This system has been modified such that there are two versions: in the original version, the tetracycline transactivator cannot bind the tet operator in the presence of tetracycline ('tet-off'). In the revised system, the transactivator can only bind the operator in the presence of tetracycline ('tet-on'). The main difficulty with this system is that the rate of tetracycline or doxycycline clearance, which can take 24 hours to 1 week, greatly affects its utility (Hasan et al., 2001; Kistner et al., 1996).

Another modification allowing temporal control of recombinase expression is the fusion of a mutant ligand binding domain of the estrogen receptor to the C-terminus of the site-specific recombinase (Brocard et al., 1997; Feil et al., 1996; Kellendonk et al., 1996; Logie and Stewart, 1995; Metzger et al., 1995; Schwenk et al., 1998). The mutated estrogen receptor is not activated by endogenous β-estradiol, but is activated by the synthetic estrogen antagonist, 4-OH tamoxifen (Feil et al., 1996). Activation of the site-

specific recombinase is dependent on administration of 4-OH tamoxifen, which results in transport of the fusion protein to the nucleus. Using a ubiquitous promoter to drive CreER^{TM} expression, efficient recombination was observed within 15 hours of administration of the activator, and peaked at 48 hours (Hayashi and McMahon, 2002). This system has not widely been used to study recombination events *in utero*, as it has been difficult to optimize the dosage required for recombination to occur and the required doses are often too close to toxic ranges (Indra et al., 1999). This system cannot be used to study recombination events in the preimplantation embryo in vivo, as administration of 4-OH tamoxifen at E3.5 blocks implantation because it interferes with the essential estrogen surge around implantation (Bloxham et al., 1977).

1.4.4 Cre Reporter Mice

Several lines of transgenic mice have been generated that are useful in studying the expression of Cre recombinase in promoter-Cre transgenic mice. These reporter mice fall into two basic categories: the single reporter system and the double reporter system (Table 1).

1.4.4.1 Single Reporter Systems

The most common design for Cre reporter constructs involves a single reporter, whereby a histochemical or fluorescent reporter gene is only expressed following Cre-mediated recombination. The basic design involves a loxP flanked stop region, followed by the reporter gene. In the presence of Cre recombinase, recombination brings the reporter gene under control of a ubiquitous promoter, allowing its expression in any cell type that has expressed Cre recombinase (Table 1).

The most commonly used single reporter line was developed by Phillipe Soriano, and was created using the “gene trap – reverse orientation splice acceptor” or Gt Rosa system (Zambrowicz et al., 1997). In this case, a cassette containing a loxP flanked stop sequence followed by the *E.coli* β-galactosidase gene was engineered to insert into a ubiquitously expressed gene by homologous recombination. In these mice, β-galactosidase is only expressed following Cre-mediated recombination (Soriano, 1999).

1.4.4.2 Double Reporter Systems

The double reporter system is particularly useful, as these mice will allow for the identification of cells that have not expressed Cre recombinase, as well as those cells that have expressed Cre recombinase. The basic system relies on having one reporter gene followed by an extensive polyA sequence, flanked by loxP sites, such that in the absence of Cre recombinase expression, only this first reporter gene is expressed. Following the second loxP site, a second reporter gene is inserted such that it is only expressed following Cre-mediated recombination. The first line of double reporter mice to be created carry a Z/AP transgene, in reference to their two histochemical reporter systems: prior to Cre excision, these mice express β-galactosidase (Z) ubiquitously (Lobe et al., 1999). Following Cre excision, human placental alkaline phosphatase (AP) is expressed.

A second line, based on this same principle, was developed using β-galactosidase (Z) and EGFP (EG) as the reporters, to give rise to Z/EG reporter mice (Novak et al., 2000). These lines of reporter mice allow for the evaluation of expression patterns in tissue specific Cre mice. Breeding of the Cre mice to a reporter line to generate double transgenic offspring allows for the visualization of Cre expression patterns using the reporter gene, often by fluorescent imaging or histochemical staining.

1.5 Cell Lineage Tracing in the Mouse

Although little direct cell lineage tracing has been done in the mouse placenta, much has been done to study cell lineages in either the earliest developmental stages or in the embryo. With advances in technology, many different methods have been used to approach questions of cell lineage in the developing mouse embryo.

1.5.1 Inert Compounds, Enzymes, Dyes and Fluorescent Markers

The earliest work attempting to directly analyze cell lineage in the developing mouse embryo involved the use of inert compounds, enzymes or dyes as markers. In this approach, a cell was injected with the marker, which can then be followed either visually in live or cultured tissues, or by histochemical staining of fixed tissues. The earliest attempts at direct lineage tracing used inert silicone fluid to study the first divisions in the preimplantation mouse embryo (for example: (Graham and Deussen, 1978; Wilson et al., 1972)). A drop of the oil would be injected deep into the cytoplasm of a single cell and

Table 1. Cre-Responsive Indicator Mice

Indicator	Transgenic or Knock In	Promoter/ Enhancer	Reporter	Reference
cAct-XStopXlacZ	Transgenic	chicken β-actin	nuclear β-gal	(Tsien et al., 1996)
floxLacZ	Transgenic	chicken β-actin	β-gal	(Akagi et al., 1997)
Cβ-STOP-lacZ	Transgenic	chicken β-actin	nuclear β-gal	(Zinyk et al., 1998)
R26R	Knock In	ROSA26	β-gal	(Soriano, 1999)
ROSA26^{flox}	Knock In	proviral ROSA26	β-gal	(Mao et al., 2001)
Z/AP	Transgenic	CAG	β-gal hPLAP	(Lobe et al., 1999)
Z/EG	Transgenic	CAG	β-gal eGFP	(Novak et al., 2000)
CAG-CAT-EGFP	Transgenic	CAG	eGFP	(Kawamoto et al., 2000)
R26R-EYFP	Knock In	ROSA26	eYFP	(Srinivas et al., 2001)
R26R-ECFP	Knock In	ROSA26	eCFP	(Srinivas et al., 2001)
ROSA26-EGFP^{f}	Knock In	ROSA26	eGFP	(Mao et al., 2001)
LSLMAP2GFP	Transgenic	chicken β-actin	MAP2-GFP	(Huang et al., 2002)

Table adapted from (Branda and Dymecki, 2004) *CAG*, chicken *β-actin* promoter linked to cytomegalovirus enhancer; β-gal, beta galactosidase; hPLAP, human placental alkaline phosphatase; EGFP, enhanced green fluorescent protein; EYFP, enhanced yellow fluorescent protein; ECFP, enhanced cyan fluorescent protein.

then could be visualized as the cells of the embryo divided in culture (Graham and Deussen, 1978). There were several limitations to this method. First, the oil drop would not be partitioned between progeny cells such that it would only mark one daughter cell following cell division, making it difficult to follow the lineage of a particular blastomere. Additionally, this technique could only be used to study the earliest cell divisions of the developing embryo, as culture techniques do not allow the *in vitro* maintenance of embryos past the blastocyst stage without losing normal cellular behaviour and positioning. A similar method was also employed using melanin granules (Copp, 1979), but this method had all the same caveats.

To overcome some of the limitations of the previous method, the injection of enzymes, dyes or fluorescent markers was used to examine questions of cell lineage. These methods were superior to the injection of inert compounds for several reasons; there was a reduction in the incidence of the marker segregating to a single daughter cell, the marker had a longer effective lifetime and the marked cells were easily detected either visually, fluorescently or histochemically (Cruz and Pedersen, 1985). Horseradish peroxidase (HRP) was commonly used as a histochemical marker. Once injected into a cell, it would partition to daughter cells, which could be identified using histochemical staining of fixed materials. The cells were injected during early development but it was found that HRP could not be detected in full term animals due to the dilution of the HRP over many cell divisions (Cruz and Pedersen, 1985). One difficulty involved in the injection of HRP was that it was not possible to immediately identify whether only one cell had been injected or if neighbouring cells had also received HRP. This led to the co-injection of readily visible markers such as rhodamine-conjugated dextran (RDX) or Fast Green, which are easily visible using fluorescent microscopy (Cruz and Pedersen, 1985). These markers could also be visualized over approximately 24 hours. However, Fast Green tended to diffuse independent of HRP due to its lower molecular weight, while RDX fluorescence would fade or become particulate within the first 24 hours (Cruz and Pedersen, 1985). Despite the improvements over the injection of inert compounds, these methods still only allowed short term examination of cell lineages and could only be performed *in vitro*.

A similar approach was attempted using carboxylated yellow green fluorescent latex microparticles to label cells (Fleming, 1987). The fluorescent latex microparticles are taken up by endocytosis and are useful for studying cell lineage up to three cell divisions after labelling. This lineage marker was found to be autonomous to the labelled cells and their daughter cells, and was not transferred to adjacent unlabelled cells, even when these cells were joined by a midbody connection, due to the collection of the microparticles in lysosomal compartments that were too large to pass through the cytoplasmic bridges (Fleming, 1987). Again, the main disadvantage of this marker is that cells can only be followed for the short term, and these manipulations can only be studied *in vitro*.

Another set of lineage markers that has been used to examine embryos in culture are the carbocyanine dyes, DiI (1, 1'-dioctadecyl-3, 3, 3', 3'-tetramethyl indocarbocyanine perchlorate) and DiO (3, 3 ′-dioctadecyloxacarbo-cyanine). These lipophilic dyes may be topically applied to single cells or groups of cells by manual application, followed by embryo culture *in vitro*. Labelled cells can still be visualized within the developing embryo after 48 hours of development *in vitro*, however persistence of labelled cells after this point is difficult to assess, because of the limited time span of normal development *in vitro* (Wilson and Beddington, 1996).

The main drawback in all of these methods of marking cell lineage was that their analysis was limited to *in vitro* analysis of very early developmental stages. In order to look at later stages of development, methods for introducing a more permanent lineage marker were required.

1.5.2 Passive Endogenous Genetic Markers

As techniques developed for the manipulation of early mouse embryos, it became possible to introduce passive markers into the genome, allowing for the study of cell lineage using more permanent markers. Genetic markers have many advantages over other labelling methods, because they are permanent, heritable and, if chosen wisely, are not likely to damage a cell or change its developmental potential (Price, 1987). Several different methods were developed to examine more permanent genetic markers. Among

these methods, the introduction of genetic material by retroviral infection has proven useful in the study of cell lineage.

Retroviral vectors have been designed to introduce foreign DNA into cells, in order to genetically mark these cells. The main advantage of this system is that it is naturally evolved, and as a result it is both highly efficient and highly accurate (Price, 1987). During retroviral infection, an exact copy of the retroviral genome is inserted into the host cell chromosome. The information for integration and expression is all contained within sequences flanking the retroviral genome (long terminal repeats (LTRs)). Based on this organization, any genetic sequence could theoretically be placed between LTRs and the virus would maintain the ability to infect a cell, integrate the DNA and transcribe the gene (Price, 1987). This naturally evolved system for transferring genes needed only minor modifications to generate a very effective approach for gene introduction. The most important modification was to render the viral genome replication-deficient, such that the DNA would be introduced into cells without subsequent replication and further infection of cells (Price, 1987). Once a retrovirus has infected a cell, the DNA it carries is stably integrated into the host chromosome, such that all progeny of the cell will carry the genetic change. Also, each retrovirus integrates randomly within the cell it has infected, marking each cell with a unique genetic identity.

Several applications of retroviral gene transfer have been applied in studying cell lineage in the mouse. Retroviruses were used to introduce a lineage marker into haematopoietic stem cells *in vitro*, which were then transferred into lethally irradiated mice, allowing the lineage of these stem cells to be tracked *in vivo* over several months (Lemischka et al., 1986). One major limitation of this approach is that even though a stem cell may only populate one compartment, it does not prove that its fate was determined or that it could not populate other compartments. In addition, the mice examined in experiments such as this are not normal animals, as they are recovering from radiation and bone marrow transplantation. A similar approach involves the injection of retroviral vectors into the living animal at low levels to cause localized infection, and then later examining cells that express the lineage marker (Price et al., 1987). This method also has several limitations. It is not easy to demonstrate that a clone of marked

cells originated from one precursor cell, although each cell would have a unique integration site, this would have to be examined using Southern blotting or by cloning of the integration site (Price et al., 1987). To study early cell lineage decisions, ES cells have been infected with retroviral vectors, and used to generate chimaeric mice by aggregating the ES cells with wild type embryos. Alternately, early preimplantation embryos can be infected *in vitro* and then introduced into pseudopregnant females, allowing for the tracking of marked cells. This approach is similar to other methods for generating chimaeric mice but less invasive as no aggregation or injection is required (Price, 1987). A general limitation of the use of retroviral vectors is that infection with the retroviral vector may result in viremia leading to non-cell autonomy and possible growth or differentiation effect (Lo et al., 1987; Soriano and Jaenisch, 1986).

1.5.3 Active Endogenous Genetic Markers

The transplantation of genetically disparate cells in early development has been used to study cell fate in several vertebrates. Perhaps best known are experiments in which quail tissues were transplanted into developing chick embryos to determine developmental potential (Hornbruch et al., 1979; Tickle et al., 1978). This approach has been modified for use in the early mouse embryo, using heterotypic transplantation of cells, which has revealed that in the epiblast, cells have a high level of plasticity and can acquire a novel cell fate after transplantation (Beddington, 1982; Parameswaran and Tam, 1995). In addition, fate mapping of the gastrulating mouse embryo has shown regionalization of the epiblast, with specific regions representing precursor populations for the major tissue types (Lawson et al., 1991; Lawson and Pedersen, 1992; Parameswaran and Tam, 1995). These features of development have allowed for the study of cell lineage in the epiblast using orthotopic transplantation of cells expressing a visible marker such as lacZ into non-expressing embryos to follow the fate of the transplanted cells (Parameswaran and Tam, 1995; Tam and Zhou, 1996). Following transplantation, embryos are allowed to develop in culture and cell fates of the transplanted epiblast can be analyzed using histochemical methods. This approach is limited by the developmental window during which embryos can be maintained in culture.

One of the most versatile systems currently being used for cell lineage tracing in the mouse involves the controlled activation of a permanent cell marker. Using the site-specific recombinases and reporter strains described in the previous section, a permanent genetic change which is heritable by all of its daughter cells can be introduced into a cell. The expression of the recombinase can be controlled in a spatial and temporal manner, either by changing the promoter and enhancer elements driving its expression, or by using one of the many inducible systems available. Since the reporter strains available express either a fluorescent or a histochemical marker in the presence of the recombinase, activation of the recombinase in a specific cell type can mark that cell and all of its progeny, and this marker can be examined at any stage of development.

This approach has many advantages for cell lineage tracing. Cells can be marked very early in development without a requirement for physical manipulation of the embryo. The genetic change introduced by the recombinase is irreversible and heritable and as a result can be examined at any stage following the recombination. Detection of the reporter gene in tissues can be done histochemically or immunohistochemically, allowing for the study of serial histological sections using markers for other cell types, to identify the contribution of marked cells to various populations.

1.6 Promoters Useful in the Creation of Trophoblast Cell-Type-Specific Cre Mice

1.6.1 Genes Expressed Throughout the Trophoblast Lineage

1.6.1.1 Keratin-18 (K18)

Keratin proteins form intermediate filaments in epithelial cells. This large protein family is subdivided into the acidic type I keratins and the basic type II keratins. Intermediate filaments are formed by the polymerization of keratin heterodimers, which always contain one type I keratin and one type II keratin, and different keratin pairs are characteristic of specific tissue types (Thorey et al., 1993). Keratin 18 (K18), a type I intermediate filament protein, and its dimerization partner Keratin 8 (K8) are the first keratins to be expressed during mouse development, beginning at the 8 cell stage (Oshima et al., 1983). In the developing placenta, K18 is expressed in all trophoblast cells derived from the TE, from the differentiation of the TE until term. K18 knockout mice are phenotypically normal because K19 appears to completely replace K18 in its absence

in vivo, resulting in normal cytoskeletal architecture (Magin et al., 1998). This replacement theory was supported by the appearance of a midgestational lethality phenotype in K18/K19 double knockout mice generated by interbreeding the single knockouts for these two type I keratins (Hesse et al., 2000).

In transgenic studies, the human K18 coding region, including 2.5 kb of 5' flanking sequence and 3.5 kb of 3' flanking sequence has been used to direct expression of K18, β-galactosidase, EGFP, Alkaline Phosphatase (AP) and Cre recombinase in epithelial tissues expressing the mouse K18 gene (Abe and Oshima, 1990; Thorey et al., 1993; Wen et al., 2003). In the initial study, a 10kb region of human K18 was found to direct expression of the human form of K18 in essentially the same spatial and temporal manner as the mouse K18 gene, with a few exceptions that had been previously described as being different between mouse and human expression patterns (Abe and Oshima, 1990). Subsequently, the *E. coli* β-galactosidase was inserted into the same 10kb sequence by homologous recombination, resulting in the removal of exons 2-7 of the K18 gene (Thorey et al., 1993). The deleted fragment of the human K18 gene was included in the construct downstream of the polyadenylation site, in an attempt to include the known *cis* acting factors. The addition of the β-galactosidase coding sequence into the K18 construct resulted in the loss of expression in all adult tissues (Thorey et al., 1993). Unexpected expression also occurred in various mesenchymal tissues not known to express K18. No β-galactosidase expression was detected in adult mouse tissues. Examination of various epigenetic changes to the human K18-β-galactosidase constructs identified a change in chromatinization, likely resulting in the silencing of these constructs *in vivo* (Thorey et al., 1993). The generation of constructs driving EGFP, AP and Cre expression differed slightly from the previous approaches, in that an internal ribosome entry site (IRES) was introduced downstream of the polyadenylation site in exon 7 of the human K18 gene, and the reporter gene coding sequence was added downstream of this IRES (Wen et al., 2003). The expression of EGFP was only verified by Northern and Western blot analysis, and was observed in the tissue types expressing mouse K18. The expression of AP was revealed in sections of embryonic mice and adult

mice by histochemical methods, and was found to be very similar to the expression of the human K18 transgene (Wen et al., 2003).

In order to express Cre recombinase in epithelial tissues, a Cre recombinase-mutant estrogen receptor fusion protein ($CreER^{T2}$) was used, rendering the activity of Cre recombinase dependent on the estrogen receptor antagonist tamoxifen. The $K18iresCreER^{T2}$ mice were examined by mating to the R26R Cre reporter line (described previously). Double transgenic animals were treated with 4-OH tamoxifen or vehicle, and Cre activity was shown to be completely dependent on 4-OH tamoxifen administration. Cre activity in the tissues was found to be mosaic, even in tissues with uniform K18 expression and in both embryonic and adult tissues (Wen et al., 2003). Given that the 4-OH tamoxifen-dependent Cre activity is not uniform, these particular strains of mice will only be useful for applications where not every cell type of a particular epithelium needs to be marked.

1.6.1.2 Adenosine Deaminase (ADA)

Adenosine deaminase (ADA) is a purine metabolic enzyme that catalyzes the irreversible amination of adenine or deoxyadenine to form inosine or deoxyinosine. As such, it is an important regulator of steady state adenosine and deoxyadenosine, both of which have toxic effects when in excess (Simmonds et al., 1978). The sequence of ADA is highly conserved from bacteria to humans (Shi et al., 1997). In humans, severe deficiency for ADA is associated with a form of severe combined immunodeficiency (SCID) (Buckley, 2004).

ADA activity has been detected in all mammalian tissue types, but levels of expression vary over a range of 1000 fold (Shi et al., 1997). In mice, the levels of ADA expression can vary over as much as 10,000 fold, with the highest levels of expression being observed in decidua, placenta, tongue, esophagus, stomach and proximal small intestine(Shi et al., 1997). The expression of ADA within the placenta is subject to developmental regulation, and high levels of expression must be maintained in the placenta in order for normal development of the embryo to occur. ADA-deficient mice die perinatally of severe liver damage, as a result of the severe disturbances in purine metabolism (Wakamiya et al., 1995). Expression of ADA within the rodent placenta is

first detected at E7.5 in the trophoblast giant cells and in diploid precursor cells within the ectoplacental cone (Shi et al., 1997). As the placenta develops, expression of ADA increases. At E13.5, ADA expression is observed in all cells of the trophoblast lineage and this expression persists until term (Shi et al., 1997).

Analysis of the ADA promoter region demonstrated that the basal promoter, plus 6.4 kb of 5' flanking sequence could direct chloramphenicol acetyltransferase (CAT) activity in transgenic mice generated by microinjection (Winston et al., 1992). A deletion series of this initial construct identified a 770 bp fragment located 5.4 kb upstream of the transcriptional start site that is able to direct high levels of CAT activity in transgenic mice (Shi et al., 1997).

1.6.2 Syncytiotrophoblast-Specific Promoters

1.6.2.1 Tissue Non-Specific Alkaline Phosphatase (TNAP)

Alkaline phosphatases (APs) are membrane-anchored enzymes found in most organisms, with the exception of some higher plants (Hoshi et al., 1997). Most organisms, including humans and rodents, express several different alkaline phosphatase genes. The APs catalyze the hydrolysis of monophosphate esters, resulting in the release of inorganic phosphate (Le Du and Millan, 2002), but the physiological role of these enzymes is not well understood.

In the mouse, at least four different alkaline phosphatase (AP) genes have been identified. Three of these alkaline phosphatase genes exhibit tissue specific expression patterns; intestinal alkaline phosphatase (IAP), placental alkaline phosphatase (PLAP) and germ cell alkaline phosphatase (GCAP). Tissue non-specific alkaline phosphatase is expressed in several different tissues during development, including the skeletal system, the gonad, the intestine, and the labyrinth layer of the placenta (MacGregor et al., 1995). Within the labyrinth, TNAP is expressed specifically in the syncytiotrophoblast cells (Smith, 1973). TNAP deficient mice survive to birth, with no obvious effect on embryonic development, including primordial germ cell migration (MacGregor et al., 1995). However, the homozygous null animals developed seizures and died perinatally at about 2 weeks of age. The lethality was subsequently found to be due to defects in the metabolism of vitamin B6 (Waymire et al., 1995).

Attempts to generate transgenic mice with a β-galactosidase gene under the control of various sequences found upstream of the TNAP locus have been mostly unsuccessful. The lack of expression from the transgenic constructs was attributed to gene silencing effects due to increased methylation of both the 5' sequences and the β-galactosidase sequence (Escalante-Alcalde et al., 1996). The generation of TNAP-Cre mice was achieved using a knock-in approach, whereby Cre recombinase was inserted into the TNAP locus by homologous recombination in ES cells, and subsequently germline chimaeras were generated (Lomeli et al., 2000). The expression of Cre in these mice was assessed by crossing with the Z/AP reporter mice. Double transgenic embryos showed specific Cre expression in the primordial germ cells (PGC) at E9.5 and E10.5. This expression was also observed at E13, along with expression in the labyrinth layer of the placenta, intestine and ventral neural tube. However, the expression in tissues other than the PGCs was observed at a much lower level than in the PGCs. Therefore, the frequency of PGC-only excision is high enough to consider these mice useful for the study of PGC, as opposed to the other tissues that normally express TNAP (Lomeli et al., 2000).

1.6.2.2 Aromatase (CYP19)

Aromatase is an important enzyme in the biosynthesis of estrogens from androgens. The aromatase cytochrome P450 enzyme is coupled to NADPH-cytochrome P450 reductase, a ubiquitous flavoprotein, in order to catalyze the conversion of C_{19} steroids (androstenedione, testosterone) to C_{18} estrogens (estrone, estradiol). In humans, aromatase deficiency is often only detected in pregnancy, based on the virilization of genetically female embryos due to increased levels of testosterones and decreased levels of estrogens in the fetal environment.

Aromatase is encoded by the *CYP19* gene, which is highly conserved in all vertebrates. In most species, including mice and other rodents, expression of aromatase is restricted to the gonads and brain (Conley and Hinshelwood, 2001). In humans, aromatase is also expressed in the syncytiotrophoblast cells of the labyrinth in the placenta, as well as adipose stromal cells, osteoblasts, fibroblasts, some vascular components and the fetal liver (Kamat et al., 1999). The expression of aromatase in these

diverse tissues is controlled by several tissue-specific promoters and a specific exon 1 (Harada et al., 2003). In humans, placental expression is initiated at a unique exon, exon I.1, located 40 bp upstream of the rest of the *CYP19* gene. The translational start site is located in exon 2, such that an identical protein is produced in all tissues despite the use of different first exons.

The placental specific exon I.1 has been studied extensively, and a region spanning -501bp to +103 bp was shown to mediate syncytiotrophoblast specific expression in transgenic mice, using a human growth hormone (hGH) fusion protein (Kamat et al., 1999). These results suggest that rodents have the trans acting factors that are necessary for placenta-specific expression of the aromatase gene. However, another group generated transgenic mice carrying a 12.7 kb construct consisting of four of the upstream exons (I.1, I.4, I.3 and PII) driving expression of a β-galactosidase reporter gene (Harada et al., 2003). In this case, although reporter gene expression was detected in the mouse placenta, its expression was not initiated at exon I.1, but rather at either exon I.4 or I.3. The difference between the two studies lies in the construct of the reporters. Of principle importance, the construct used in first study contained only 500 bp of sequence upstream of exon I.1 (Kamat et al., 1999) while the construct generated for the second study included 2400 bp of promoter region upstream of exon I.1 (Harada et al., 2003). Harada et al. (2003) suggest that silencer elements may reside outside of the 500 bp minimal region used in the previous study, that result in silencing of the expression from exon I.1 in mice carrying larger genomic regions. An alternate possibility is that mice may lack transcriptional elements that are required for the tissue-specific expression of aromatase in the placenta, while still having factors that loosely enhance the transcription of aromatase from all of the exons I in the mouse (Harada et al., 2003).

1.6.3 Spongiotrophoblast/Glycogen Trophoblast Specific Promoters

1.6.3.1 Trophoblast-specific protein alpha (Tpbpa)

Trophoblast-specific protein alpha (Tpbpa) was identified in a differential screen seeking to identify genes that are expressed specifically in the trophoblast lineage (Lescisin et al., 1988). The identified cDNA (cDNA 4311) was found to encode a protein

that lacks a transmembrane domain and contains a putative signal peptide at the amino terminal end of the protein, suggesting it is a secreted protein (Iwatsuki et al., 2000; Lescisin et al., 1988). Very little is known about the function of this protein. However, it is predicted to be an inhibitor of the C1A family of cysteine peptidases, which includes many of the cathepsin proteins (Deussing et al., 2002). The assignment of this putative function is based on the amino acid sequence similarity of Tpbpa to the proregion of the cysteine peptidases, as well as the mapping of the murine genes for *Tpbpa* and *Tpbpb* to a dense cluster of placenta-specific cysteine peptidase genes (Deussing et al., 2002). Additionally, the cytotoxic T-lymphocyte antigen (CTLA)-2a and -2b genes map to the same cluster (Deussing et al., 2002) and are also classified as cysteine peptidase inhibitors (Cheon et al., 2004; Delaria et al., 1994; Iwatsuki et al., 2000). The *CTLA-2*s have been shown to inhibit cathepsin L-like cysteine peptidases *in vitro* (Delaria et al., 1994). As the *CTLA-2s* share similarity in sequence to the *Tpbp* genes (Deussing et al., 2002), it is possible they also share similar functions. As of yet, no human orthologs of the *Tpbp* genes, the *CTLA-2* genes, or the murine placenta-specific cysteine peptidase genes have been identified (Deussing et al., 2002).

The expression of murine *Tpbpa* is restricted to the placenta, as no expression has been observed other tissues of the developing embryo or adult animal. *Tpbpa* mRNA can be detected beginning at E6.5 and by E7.5 is expressed in a small subset of cells in the EPC. At E8.5 until E10.5, a subset of cells at the mesometrial end of the EPC express *Tpbpa,* as well as isolated cells within the decidua. From E13.5 until term, *Tpbpa* expression is seen within the spongiotrophoblast region of the placenta, with no expression observed in TGCs or within the labyrinth layer (Calzonetti et al., 1995; Carney et al., 1993; Lescisin et al., 1988).

Transgenic mice have been developed using various lengths of 5' flanking sequence in an attempt to direct spongiotrophoblast specific transgene expression. When 5.4 kb of 5' flanking sequence and the first intron of the *Tpbpa* gene are placed upstream of a β-galactosidase gene, appropriate spongiotrophoblast specific expression was observed in the developing placenta (Calzonetti et al., 1995). Further promoter deletions identified a 340 bp region in the 5' flanking sequence that is sufficient, with the

endogenous minimal promoter, to direct lacZ expression to the spongiotrophoblast region of the placenta (Calzonetti et al., 1995).

1.6.4 Trophoblast Giant Cell Specific Promoters

1.6.4.1 Renin 1 (Ren-1)

Renin is an aspartyl protease that is best known for its role in maintaining blood pressure and electrolyte homeostasis, as part of the renin-angiotensin system (Sigmund and Gross, 1991). Active renin is produced and stored in the juxtaglomerular (JG) cells of the adult kidney, where it is then secreted into the bloodstream. All inbred strains of mice have at least one locus encoding renin (*Ren-1*) on chromosome 1, while some strains also harbour a relatively recent duplication of this locus, encoding a second renin gene, *Ren-2* (Fabian et al., 1989). Two different alleles of *Ren-1* have been identified in different strains carrying a single locus encoding renin; *Ren-1*^{c} and *Ren-1*^{d}. All three genes are highly similar in both nucleotide sequence and genomic organization (Fabian et al., 1989). The *Ren-1*^{d} locus has been knocked out in 129 mice, carrying both *Ren-1*^{d} and *Ren-2* encoding genes (Clark et al., 1997). The *Ren-1*$^{d\,-/-}$ mice showed abnormal kidney morphology and a complete absence of granulation in the JG cells, as well as sexually dimorphic hypotension with female mice having significantly decreased mean arterial pressure. *Ren-2* has also been knocked out. These mice were healthy and viable, with no changes to mean arterial pressure in adult mice (Sharp et al., 1996). The only observable change was a decrease in the concentration of circulating inactive prorenin, and an increase in the concentration of circulating active renin. Thus, *Ren-2* is not required for maintaining homeostasis. As it had been previously found that *Ren-2* is likely to have different glycosylation potential, due to the loss of several asparagine-linked glycosylation sites (Sigmund and Gross, 1991), the *Ren-2* protein may not be active in the mouse.

The expression of renin in the developing and adult mouse has been studied in great detail. In mice, renin expression is first detected at about E14, in the forming renal arteries, and becomes progressively restricted to more distal areas of the arteries as the kidney develops (Sigmund and Gross, 1991). As the vascular smooth muscle cells differentiate, they stop producing renin until only the specialized smooth muscle cells of

the JG express renin in the most distal regions of the intrarenal arteries (Jones et al., 1990). These vascular smooth muscle cells of the intrarenal arteries appear to retain the ability to express renin, and can be recruited to a renin-expressing phenotype in response to physiological cues or the administration of angiotensin-converting enzyme inhibitors (Petrovic et al., 1996).

Extrarenal sites of renin expression have also been observed in the mouse. Renin expression has been observed in the adrenal gland during early development; this expression is strong at E14, but becomes progressively weaker until it is no longer apparent by E17 (Fabian et al., 1989; Jones et al., 2000). Renin expression can also be detected in the gonadal arteries; this expression continues at least into the early neonatal period (Jones et al., 2000). In the adult, renin expression is detectable in the submandibular gland in a sexually dimorphic manner; male mice express renin at this site, while female mice do not (Jones et al., 2000).

The components of the renin-angiotensin system have been observed in the pregnant human uterus. However, the localization of the site of renin production has long been disputed (Hanssens et al., 1995; Ihara et al., 1987; Poisner et al., 1981; Shaw et al., 1989). In the mouse, renin expression has been localized to the trophoblast giant cells, beginning at about E13 and continuing until parturition (Jones et al., 2000).

Extensive studies have been undertaken to examine the regulation of the renin genes. Many of these studies involve the introduction of renin 5' flanking sequence linked to a variety of reporters into either cell culture systems or transgenic mice. These studies have shown that the addition of either 0.45 kb or 2.5 kb of 5' flanking sequence linked to the SV40 large T antigen (TAg) exhibited inappropriate expression patterns in transgenic mice. However, if 4.6 kb of upstream sequence was included, correct tissue- and cell-specific expression could be achieved (Sola et al., 1989). Further examination of this upstream region reduced the required 5' flanking sequence to 4.1 kb (Petrovic et al., 1996). A 241 bp fragment from -2625 to -2866 was also identified that, in conjunction with the basal renin promoter, retained full activity in cell culture in an orientation independent manner. This response is typical of a classical enhancer element. The 4.1 kb 5' flanking sequence was further tested *in vivo* by linking this to GFP (Jones et al.,

2000). When introduced into mice, this construct directed high levels of GFP expression in the same tissue- and cell-specific manner of renin expression beginning at E13 and continuing into adulthood. Additionally, treatment of these transgenic mice with an angiotensin-converting enzyme inhibitor induced recruitment of vascular smooth muscle cells to a renin expressing phenotype (Jones et al., 2000). Thus, 4.1 kb of 5' flanking sequence of the Ren-1 locus is sufficient to drive strong, tissue- and cell-specific expression in transgenic mice.

1.6.4.2 Placental Lactogen 1(PL1) and Placental Lactogen 2 (PL2)

Placental lactogen (Pl) 1 and Pl2 are members of the prolactin/growth hormone family (Colosi et al., 1987; Jackson et al., 1986). These molecules both bind the prolactin receptor with high affinity and are involved in the maintenance of ovarian progesterone production and the development of the maternal mammary glands for postpartum lactation (Ogren and Talamantes, 1988). Both *Pl1* and *Pl2* are expressed exclusively from trophoblast giant cells in the mouse placenta (Faria et al., 1991). Expression of *Pl1* begins at about E9, while at E10 both *Pl1* and *Pl2* are expressed and by E11 and afterward, *Pl2* is expressed exclusively. This pattern of expression coincides with a midgestation shift in placental hormone expression that includes changes from expression of Pl1, proliferin and the proliferin-like proteins (PLP) A and E to Pl2 and proliferin-related protein (PRP) (Carney et al., 1993; Faria et al., 1991). These changes in gene expression represent a molecular basis for identifying distinct stages of trophoblast giant cell differentiation (Shida et al., 1992).

The regulation of the *Pl1*gene has been studied in the rat choriocarcinoma cell line Rcho-1 (Shida et al., 1993). This study identified a 274 bp sequence extending upstream from the transcriptional start site that was sufficient to direct maximal expression in this trophoblast culture model. This sequence and another larger upstream sequence (2700 bp) were unable to direct expression in a variety of other cell types in culture (Shida et al., 1993). The expression of these upstream sequences has yet to be investigated in transgenic mice. Initial attempts by our lab to create mice expressing Cre recombinase under the control of the *Pl1* promoter have been unsuccessful, even using 10 kb of 5' upstream sequence (H. Yamamoto and J.C. Cross, unpublished results).

The regulation of the *Pl2* gene has also been studied (Shida et al., 1992). A 2.7 kb region of upstream sequence was linked to the SV40 T antigens and used to generate transgenic mice by microinjection. Three transgenic lines were generated, and placenta specific expression was observed in all of these lines. The pattern of T antigen expression was identical to that of the endogenous *Pl2* in the transgenic mice. A deletion construct containing only 569 bp of upstream sequence was unable to direct placenta specific expression. These results suggest that the 2.7 kb upstream sequence could be useful for directing Cre recombinase expression to the trophoblast giant cells of the mouse placenta.

1.7 Conclusion

The use of the Cre-loxP system and reporter mice allows for the introduction of permanent, heritable genetic changes that can be followed throughout development. This enables the study of cell lineage questions in vivo in tissues that were not previously studied with traditional techniques. We have generated mice in which Cre recombinase expression is under the control of the *Tpbpa* promoter, and is therefore expressed in the EPC and spongiotrophoblast region of the placenta. By crossing these mice to the Z/AP reporter line (Lobe et al., 1999), we were able to examine the fate of *Tpbpa*-expressing cells *in vivo*. The development of other trophoblast-specific Cre mice will allow for the examination of other cell lineage relationships in the developing placenta.

CHAPTER TWO:

PLACENTAL CELL LINEAGE TRACING USING CRE RECOMBINASE TRANSGENIC MICE

The studies in this chapter were performed by the author except that plasmid generation (not including the pGEM-Cre plasmid), the RNase protection assays and the EGFP fluorescence image shown in Figure 4 were performed by Dr. Hideyuki Yamamoto.

2.1 Introduction

The cell lineage relationships in the placenta have thus far largely been assumed based on indirect evidence. The methods used for cell lineage tracing were not generally amenable to the study of development *in utero.* However, the use of site specific recombinases both *in vitro* and *in vivo* has allowed for the development of cell lineage tracing methods in tissues that were previously unavailable for direct studies, including the placenta. Cell-type specific expression of Cre recombinase can be used to make a permanent, heritable and readily detectable genetic change in specific cell populations, simply by crossing Cre transgenic mice to one of the many available Cre reporter lines. The most important feature of this system is the Cre expression in a cell will result in the expression of the reporter in all cells descendent from the recombined cell. This feature is based on the use of ubiquitous promoters to drive the reporter expression, such that even in the absence of Cre, cells that have arisen from a cell that has undergone recombination will express the reporter.

The trophoblast lineage is the main component of the placenta and is derived from the first cell lineage to be specified during development. In the mouse, the trophectoderm is specified at E3.5, when the blastocyst is formed. The trophectoderm surrounds the blastocyst and the inner cell mass (ICM), and already has two different fates at this stage. At implantation, the cells that are not in direct contact with the ICM cease proliferating and terminally differentiate to form primary trophoblast giant cells (Cross, 2000). This initial connection between the trophoblast and the maternal decidua is essential for the early nutrition of the embryo and failure to establish this connection can lead to nutritional insufficiency and peri-implantation death (Copp, 1995). The polar trophectoderm cells that overlie the ICM continue to proliferate and will give rise to the trophoblast cell types of the placenta (Cross, 2000). The cell lineage relationship between the trophoblast cells beyond this point are much less clear, and have mostly been inferred from indirect evidence. The polar trophectoderm gives rise to two distinct cell populations, the extraembryonic ectoderm and the ectoplacental cone.

Cells of the extraembryonic ectoderm give rise to the chorion, and then to the syncytiotrophoblast cells, which form the bulk of the labyrinth layer of the placenta. Evidence for this lineage is indirect and primarily based on the restricted expression of certain genes within these cell types (Cross, 2000). Cells of the EPC give rise to the secondary trophoblast giant cells and the spongiotrophoblast and glycogen trophoblast cells. The evidence for this lineage is based on both gene expression patterns and explant cultures of the EPC. Expression of the spongiotrophoblast/glycogen trophoblast specific marker, *Tpbpa* begins in the EPC at E7.5 in a small subset of cells and expression increases as placental development proceeds (Lescisin et al., 1988). At E10.5, when a mature placenta has been formed, *Tpbpa* expression is restricted to the middle spongiotrophoblast layer and later to the glycogen trophoblast cells (Calzonetti et al., 1995; Lescisin et al., 1988). The secondary trophoblast giant cells arise from the differentiation of cells at the tip of the EPC, at the furthest distance from the embryo, and later from the spongiotrophoblast. Additional evidence supporting the formation of these cell types from the EPC arises from the explant culture of EPC cells (Carney et al., 1993). When these cells are dissected and placed in culture, they pass through a stage where they express *Tpbpa* and within 2 days begin to express trophoblast giant cell marker genes, including placental lactogen-I (*Pl1)* and proliferin (*Plf).*

The developing vasculature of the placenta, which facilitates the transfer of nutrients and gases to the fetus, is influenced by the movement of trophoblast cells within the placenta (Adamson et al., 2002). The maternal blood enters the placenta through spiral arteries that form in the implantation site by angiogenesis. These arteries are invaded by trophoblast cells, which displace the endothelial cell lining and reduce maternal control of blood flow to the placenta (Hemberger et al., 2003). These cells express markers of trophoblast giant cells. As the spiral arteries approach the outer trophoblast giant cell layer, they converge to form large central canals that flow toward the base of the placenta, where maternal blood then passes into very small sinusoid spaces that are separated from the fetal blood spaces by three layers of trophoblast cells, two of which are syncytiotrophoblast (Adamson et al., 2002). Fetal blood enters the

placenta through a single umbilical artery, which branches extensively to form a dense network for exchange of nutrients, gases and waste.

In order to examine the question of cell lineage in the mouse placenta, we have exploited the features of Cre recombinase reporter lines. We have generated mice in which Cre recombinase is expressed in the EPC and spongiotrophoblast, under the control of the *Tpbpa* promoter. These mice have been crossed to the Z/AP reporter line (Lobe et al., 1999) to examine the fate of *Tpbpa*-expressing cells *in vivo*.

2.2 Materials and Methods

2.2.1 Plasmids

Transgenic constructs. In order to generate an expression cassette for the bicistronic expression of both Cre recombinase and EGFP, several steps were required. First, an XbaI/blunted to BglII fragment of pCAGGS (Niwa et al., 1991) containing the Cre recombinase coding sequence was subcloned into pUC19 to generate the 4000bp pCre-1 plasmid. pCre-1 was digested with BstI, blunted and further digested with EcoRI to liberate the Cre coding sequence, which was then inserted into pIRES2-EGFP (Clontech) between the blunted XhoI site and the EcoRI site, to generate the 8355 bp plasmid pCIG. To create a more versatile plasmid containing the Cre-IRES-EGFP construct, a PstI fragment liberating the entire Cre-IRES-EGFP construct was subcloned into pBS-KS+ (Stratagene) to generate pCIG-BSK1.

To generate a construct in which the Cre-IRES-EGFP bicistronic cassette would be specifically expressed in the spongiotrophoblast region of the mouse placenta, it was placed under the control of the *Tpbpa* promoter (Calzonetti et al., 1995). This was accomplished by sucloning a 6991 bp EcoRI fragment of pKS4311 (provided by the laboratory of J. Rossant) containing 5.4 kb of 5' flanking sequence as well as the first intron of the Tpbpa gene into the EcoRI site of pCIG-BSK1 to generate the 13167 bp plasmid, pTpbpa-CIG.

Riboprobe plasmids. Several constructs were generated for the creation of riboprobes for the detection of mRNA, either by RNase protection or in situ hybridization. To generate a riboprobe for the detection of *Tpbpa* RNA in the RNase protection assay, the

plasmid p4311-214 was generated. This plasmid was created by cloning a portion of the *Tpbpa* cDNA by RT-PCR from total placental RNA using the primers JCC612 (5' GCAAGAGCAGA AGGATAAAGAAGTTCTCATA 3') and JCC613 (5' TGGCTGTGGTTTGTTTTCCTCCTC 3') and inserting the resultant product into the pGEM-T vector (Promega). To generate a riboprobe for the detection of *EGFP* RNA in the RNase protection assay, the plasmid pEGFP-263 was generated. This plasmid was created by first generating a fragment of the *EGFP* cDNA by PCR amplification from the plasmid pCIG-BSK1 using the primers JCC381 (5'TTCAAGGACGACGGCAA CTACAAGA 3') and JCC382 (5' GGGGGTGTTCTGCTGGTAGTGGTC 3'). This fragment of the *EGFP* cDNA was then ligated into the pGEM-T vector (Promega).

To generate a riboprobe for Cre recombinase, a 1.1kb EcoRI fragment from pTurbo-Cre containing the complete NLS-Cre sequence was inserted into the EcoRI site of the pGEM-7Zf vector (Promega). This created the plasmid pGEM-Cre, which was used to generate antisense riboprobe for in situ hybridization.

2.2.2 Generation of Tpbpa-CIG transgenic mice and breeding

The *Tpbpa-CIG* construct was prepared for microinjection by PvuII digestion of the plasmid pTpbpa-CIG, followed by isolation of the 10 kb fragment containing the intact construct without as little flanking bacterial sequence as possible. The DNA was purified, quantified and diluted to a concentration of 1-2 ng/μl prior to being sent to the transgenic core at the Centre for Molecular Medicine and Therapeutics (Vancouver, British Columbia), where it was used to create transgenic mice by microinjection. Three founder transgenic males were outcrossed to CD1 females to produce progeny that were heterozygous for the *Tpbpa-CIG* transgene.

2.2.3 Genotyping

The initial genotyping of *Tpbpa-CIG* transgenic mice was based on PCR detection of the EGFP coding region using the primers JCC381 and JCC382 (primer sequences shown above). The components of the PCR reaction were Fisher 'B' 10X PCR buffer (Fisher Scientific), 25mM $MgCl_2$, 5pmol dNTPs, 10 pmol each of JCC381 and JCC382, 1U Taq polymerase and 1μl of DNA in a final volume of 25 μl. The PCR reaction was conducted as follows: 94^{o}C for 4 minutes followed by 30 cycles of 94^{o}C for 1 second,

65°C for 1 minute, and 72°C for 1 minute. This reaction generates an approximately 250 bp PCR fragment if the *EGFP* coding region is present in the DNA sample.

An alternative method for genotyping the *Tpbpa-CIG* transgenic mice was based on detection of region encoding *Cre* using the primers JCC383 (5'GTTCGCAAGAAC CTGATGGACA 3') and JCC384 (5' CTAGAGCCTGTTTTGCACGTTC 3'). The components of the PCR reaction were Fisher 'B' 10X PCR buffer (Fisher Scientific), 25 mM $MgCl_2$, 5 pmol dNTPs, 10 pmol each of JCC383 and JCC384, 1U Taq polymerase and 1 μl of DNA in a final volume of 25 μl. The PCR reaction was conducted as follows: 94°C for 5 minutes then 30 cycles of 94°C for 1 minute, 54°C for 1 minute, 72°C for 1 minute followed by a 5 minute extension at 72°C. This reaction generates a ~300 bp PCR fragment if the Cre recombinase coding region is present in the DNA sample. This method was used for routine genotyping of the *Tpbpa-Cre* mice.

Genotyping for *Z/AP* transgenes was carried out on ear punches (for adult mice) or on small tissue samples (for embryos, a small piece of tail was cut for genotyping) as described previously (Lobe et al., 1999). Samples were rinsed in PBS, fixed for 15 minutes in 0.2% glutaraldehyde in PBS, and washed three times for 5 minutes in PBS. Samples were then stained with lacZ stain (0.5mg/ml 4-chloro-5-bromo-3-indolyl-β-D-galactopyranoside (X-gal), 5mM potassium ferrocyanide and 5mM potassium ferricyanide in lacZ wash buffer) at room temperature with protection from light.

PCR genotyping of Gt Rosa Sor ^{tm1sor} mice (Soriano, 1999) was conducted using the primers JCC745 (5'GCGAAGAGTTTGTCCTCAACC 3'), JCC746 (5' GGAGCGGGAGAAATGGATATG 3') and JCC747 (5' AAAGTCGCTCTGAGTTGT TAT 3'). These primers were designed by the lab of Dr. Phillipe Soriano (Friedrich and Soriano, 1991; Zambrowicz et al., 1997). The PCR reaction was conducted using Epicentre FailSafe 2X PCR Premix Buffer B (Epicentre Technologies), 10pmol each of JCC745, JCC746 and JCC747, 0.5U Taq polymerase and 1μl of DNA in a final volume of 25μl. The PCR reaction was conducted as follows: 94°C for 3 minutes then 35 cycles of 94°C for 30 seconds, 67°C for 1 minute, 72°C for 1 minute followed by a 10 minute extension at 72°C. This three primer system generates a ~550 bp wild type band and a ~300 bp transgenic band.

2.2.4 RNase Protection Assay

RNase protection assays were performed using the Ambion RPA III kit (Ambion), generally as described in the kit instruction manual but with minor modifications as follows. All required reagents are included in the RPA III kit. Briefly, *Tpbpa* and *EGFP* riboprobes generated using the plasmids p4311-214 and pEGFP-263 were added to 10-20μg of total RNA isolated from embryos or placentas from *Tpbpa-Cre* transgenic mice and dried in a speed-vac for 15-30 minutes. To this, Hybridization III buffer was added and the solution was heated to 85-95^{o}C for 4 minutes and then incubated at 43^{o}C for a minimum of 16 hours. RNase solution (RNaseA/RNase T1 mix and RNase Digestion Buffer) was added; samples were mixed well and incubated at 37^{o}C for 30 minutes. RNase inactivation buffer was added. Samples were incubated at -20^{o}C for 15 minutes, followed by a 15 minute centrifugation at top speed. The supernatant was carefully removed and tubes were dried for 5 minutes. Pellets were resuspended in Gel Loading Buffer II and heated to 95^{o}C for 3 minutes before being run on an acrylamide gel at 40 watts for 1.5-2 hours. Gels were dried, exposed overnight at -85^{o}C and films were developed.

2.2.5 Tissue collection for whole-mount staining or for sectioning

Embryos and extraembryonic tissues were dissected at various gestational ages. Noon of the day on which a vaginal plug was detected was considered to be E0.5. Fixation of tissues for whole mount histochemical staining was performed as described previously (Lobe et al., 1999). Briefly, small embryos and placentas (harvested on or before E9.5) to be used for X-gal staining were fixed in lacZ fix (0.2% glutaraldehyde, 50mM EGTA, pH 7.3, 100mM $MgCl_2$ in PBS) overnight at 4^{o}C. Embryos and placentas harvested after E9.5 for X-gal staining were fixed in 2% paraformaldehyde, 0.2% glutaraldehyde in PBS overnight at 4^{o}C. Embryos and placentas to be used for alkaline phosphatase staining were fixed in 0.2% glutaraldehyde, 5mM EGTA, pH 7.3, 10mM MgCl2, 0.02% Nonidet-P40, 0.01% sodium deoxycholate in phosphate buffered saline overnight at 4^{o}C.

Placentas to be used for routine histology, *in situ* hybridization or immunohistochemistry were fixed overnight in 10% neutral buffered formalin (10%

formaldehyde, 33mM sodium phosphate, monobasic, 45mM sodium phosphate, dibasic in distilled H_20) and embedded in paraffin. Sections were cut at 8μm.

2.2.6 Whole-mount β-galactosidase staining and embedding

Whole-mount β-galactosidase staining was performed as described previously (Lobe et al., 1999). Briefly, fixed samples were washed three times in lacZ wash buffer (2mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% Nonidet-P40 in PBS), for 20 minutes. Samples were stained in lacZ stain (0.5mg/ml 4-chloro-5-bromo-3-indolyl-β-D-galactopyranoside (X-gal), 5mM potassium ferrocyanide and 5mM potassium ferricyanide in lacZ wash buffer) at room temperature for 30 minutes to overnight, with shaking and protection from light. Once staining was complete, samples were washed three times for 10 minutes in PBS and stored in lacZ wash buffer at 4^oC.

Stained tissues were prepared for embedding using a modified procedure due to the increased fragility of the tissues as a result of the processing for staining. Samples were dehydrated through an ethanol series (30%, 50%, 70%, 85%, 95% and 100% in PBS) for 5 minutes each, followed by two 15-minute washes in xylene. Samples were paraffin-embedded and sections were cut at 8μm.

2.2.7 Whole-mount alkaline phosphatase staining and embedding

Whole-mount alkaline phosphatase staining was performed as described previously (Lobe et al., 1999). Briefly, fixed samples were rinsed in PBS then endogenous alkaline phosphatase activity was inactivated by incubation at 75^oC for 30 minutes. Samples were rinsed in PBS, and washed in AP buffer (100mM Tris-HCl, pH 9.5, 100mM NaCl, 10mM $MgCl_2$ in H_20) for 10 minutes. Samples were stained with BM Purple AP substrate, precipitating (Roche) at 4^oC for 0.5 to 36 hours. Once staining was complete, samples were washed extensively in 0.1% Tween-20, 2mM $MgCl_2$ in PBS and stored at 4^oC.

Stained tissues were prepared for paraffin embedding as described for tissues stained for β-galactosidase activity.

2.2.8 In situ hybridization

Histological sections prepared as described above were either stained with Harris' haematoxylin and eosin (Sigma) or subjected to in situ hybridization. Digoxygenin-

labeled antisense riboprobes for *Tpbpa* (Lescisin et al., 1988), *Cre* or *Plf* (Carney et al., 1993; Linzer et al., 1985) were prepared according to the manufacturer's instructions, using an RNA transcription kit (Roche). In situ hybridizations were carried out overnight at 53^{o}C using standard procedures (Komminoth et al., 1992). Signal detection was carried out using an anti-digoxygenin alkaline-phosphatase conjugated antibody (Roche) at a 1:800 dilution, and staining was performed using NBT/BCIP to effect. Sections were counterstained with nuclear fast red (Dako).

2.2.9 Immunohistochemistry

Histological sections prepared as described above were also used for immunohistochemical detection of the human placental alkaline phosphatase protein. Sections were first prepared by removal of the paraffin wax. Samples were soaked in xylene. The slides were then rehydrated through a series of ethanol at decreasing concentrations, followed by PBS. Endogenous peroxidase activity was quenched by soaking the slides in 4% H_20_2 in PBS for 30 minutes at room temperature. An antigen retrieval step was carried out whereby sections were incubated in 1mg/ml trypsin (1 mg trypsin tablets (Sigma) were dissolved in 1 ml of water) for 5 minutes at room temperature. In order to block non-specific binding of the secondary antibody, samples were incubated in 5% normal goat serum (Cederlane) in PBS for 45 minutes at room temperature. Detection of the hPLAP protein was conducted using a rabbit monoclonal antibody (PLAP clone SP15, Labvision Corporation). The primary antibody was diluted to a concentration of 1:50 in 1% BSA in PBS. Sections were incubated at 4^{o}C overnight. A horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (Santa Cruz) was diluted 1:200 in 1% BSA in PBS. Sections were incubated in secondary antibody for 1 hour at room temperature. The HRP was detected using the DAKO Liquid DAB+ Chromagen System (Dako), which is a substrate system for the visualization of HRP activity. Sections were incubated in substrate to effect, and counterstained with nuclear fast red (Dako) prior to mounting.

2.2.10 Statistical Analysis

Data are presented as proportions ± standard error of the mean. A Chi square test was used for comparisons between the observed inheritance of the transgene and values

expected assuming normal Mendelian inheritance. An unpaired Student's *t* test was used for comparisons between the three transgenic lines. Probability values of $P < 0.05$ were considered significant.

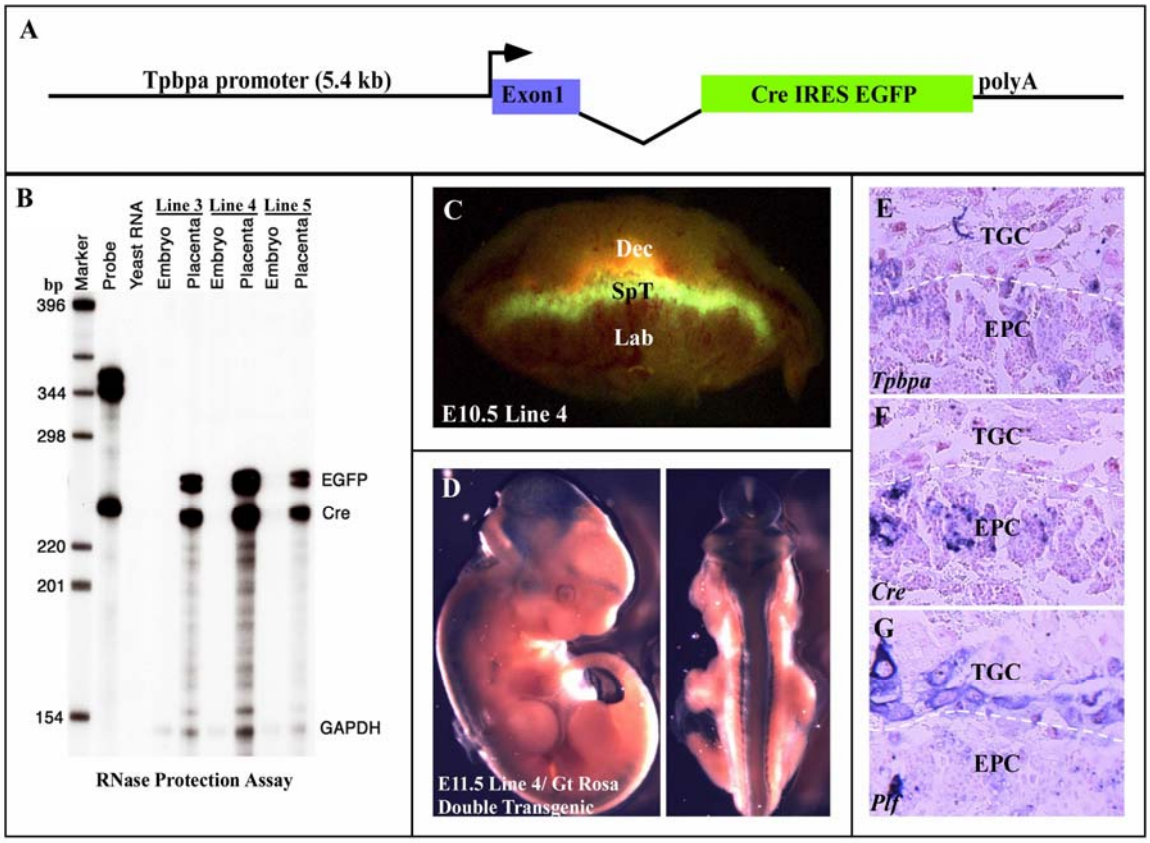
2.3 Results

2.3.1 The *Tpbpa* promoter can direct placental-specific expression of Cre and EGFP

Previous studies identified 5.4 kb of *Tpbpa* 5' flanking sequence that, along with the first intron of the coding region, could direct high level expression in the developing mouse placenta in a pattern that recapitulates the endogenous pattern of expression of the gene (Calzonetti et al., 1995). This region was linked to a CreIresEGFP (CIG) cassette (Figure 4A). The CIG cassette allows for the bi-cistronic transcription of both Cre recombinase and EGFP under the control of the same promoter. The *Tpbpa-CIG* construct was used to generate transgenic mice by microinjection. Of 31 pups, 11 (35.5%) were found to carry the *Tpbpa-CIG* transgene by PCR genotyping for the *EGFP* coding sequence. Of these 11 mice, 4 were females and were excluded from further study. The 7 male founder mice carrying the transgene were outcrossed to CD1 females, and embryos and placentas were collected at various stages to assess for Cre recombinase and EGFP expression. Total RNA was collected for individual embryos and placentas of offspring that were found to have inherited the transgene based on PCR genotyping. RNase protection assays demonstrated that the *Cre* and *EGFP* mRNAs were present in total RNA from the placenta, but not in total RNA from the fetus at E14.5 in three of the founder lines designated Line 3, Line 4 and Line 5 (Figure 4B). Further examination of the expression of the transgene (described below) revealed ectopic expression of the *Tpbpa-CIG* transgene in the developing central nervous system in offspring from Line 4 (Figure 4D), but not in Lines 3 and 5 (not shown).

Fluorescent microscopy was also used to visualize the expression of EGFP in the placentas and embryos carrying the *Tpbpa-CIG* transgene. EGPF was readily visible in the placenta, in a region corresponding to the spongiotrophoblast, in all three transgenic lines (Figure 4C, and data not shown). The fluorescence could be observed in the developing placenta as early as E9.5 (data not shown), but could not be visualized at any

Figure 4. **Generation of spongiotrophoblast specific Cre mice**. (A) Schematic diagram of the *Tpbpa-CIG* transgene. (B) RNase protection assay of total RNA isolated from embryos and placentas from each of the transgenic lines. Yeast RNA was used as a negative control. (C) EGFP fluorescence in the spongiotrophoblast region of the placenta at E10.5 (Line 4). (D) Ectopic transgene expression in the embryo of Line 4 transgenic mice. Shown is a *Tpbpa-CIG* and Gt Rosa Sor ^{tm1sor} double transgenic embryo at E11.5. (E-G) Expression of Cre recombinase is limited to *Tpbpa*-expressing cells at E9.5. Sections shown are from Line 3. *In situ* hybridization for Tpbpa (E), Cre (F) and Plf (G). Dec, decidua; EGFP; enhanced green fluorescent protein; EPC, ectoplacental cone; IRES, internal ribosome entry site; Lab, labyrinth; SpT, spongiotrophoblast; TGC, trophoblast giant cell.


A
Tpbpa promoter (5.4 kb)
Exon1
Cre IRES EGFP
polyA
B
Marker
Probe
Yeast RNA
Line 3
Line 4
Line 5
Embryo
Placenta
Embryo
Placenta
Embryo
Placenta
bp
396
344
298
220
201
154
EGFP
Cre
GAPDH
RNase Protection Assay
C
Dec
SpT
Lab
E10.5 Line 4
D
E11.5 Line 4/ Gt Rosa
Double Transgenic
E
TGC
EPC
Tpbpa
F
TGC
EPC
Cre
G
TGC
EPC
Pif

earlier stages. The visualization of EGFP was found to correlate exactly with offspring found to carry the transgene by PCR genotyping.

The expression of *Tpbpa, Cre* and *Plf* in the developing placenta was examined by in situ hybridization at E9.5 in all three lines. Both *Tpbpa* and *Cre* mRNAs were detected in cells at the tip of the EPC, just inside the *Plf* expressing trophoblast giant cell layer (Figure 4E-G, and data not shown). These observations indicate that *Tpbpa* and *Cre* are expressed in the same population of cells.

Routine PCR genotyping of offspring of the three *Tpbpa-CIG* lines revealed that the transgene is inherited at Mendelian ratios (Table 2). These results demonstrate that the transgene does not have a detrimental effect on development.

2.3.2 Activity of Tpbpa-CIG during development in Line 3

In order to examine the activity of the *Tpbpa-CIG* transgene, male transgenic mice from each founder line were crossed to females carrying the *Z/AP* reporter transgene (Lobe et al., 1999) in order to generate double transgenic offspring which could be examined by histochemical staining. Embryos and placentas were dissected at various developmental stages and whole implantation sites (E8.5) or placentas were split in half. One half of the placenta was stained for β-galactosidase activity, indicating cells which had not undergone Cre-mediated recombination. The embryo and the other half of the placenta were stained for alkaline phosphatase activity, indicating cells which had undergone Cre-mediated recombination (Figure 5A). Alkaline phosphatase activity was never observed in mice that were not carrying both transgenes.

At the whole mount level, the activity pattern of the *Tpbpa-CIG* transgene in the placenta for Line 3 essentially recapitulated the expression of endogenous *Tpbpa* (Lescisin et al., 1988) (Figure 5A). However, the onset of expression may be slightly delayed in this line as expression was only evident in a very small subset of cells at the tip of the EPC by E8.5 (Figure 5B), whereas expression can usually be detected in more cells at this stage (Lescisin et al., 1988; Scott et al., 2000; Wessells et al., 2000). The expression pattern was more widespread in the EPC by E9.5. By E11.5, alkaline

Table 2. PCR genotyping of *Tpbpa-CIG* transgenic mice

	Cre Positive	Total animals	% Cre positive	P-value
Line 3	51	107	47.7%	P>0.05
Line 4	85	155	54.8%	P>0.05
Line 5	73	128	57.0%	P>0.05

phosphatase activity appeared to be restricted to the spongiotrophoblast region of the placenta. This restricted expression was also observed at E14.5.

2.3.3 Activity of Tpbpa-CIG during development in Line 4

The expression of Line 4 in the placenta was essentially indistinguishable from the expression pattern observed for Line 3. However, this line also exhibited ectopic transgene activity in the embryo proper. This expression was observed in the hindbrain and somites of the developing embryo at E11.5 and E14.5 (Figure 4D, and data not shown), but was only observable after prolonged staining of the embryo in BM Purple alkaline phosphatase substrate (7-10 days).

2.3.4 Activity of Tpbpa-CIG during development in Line 5

Tpbpa-CIG Line 5 demonstrated a different pattern of activation in the developing placenta at early time points. At E8.5, widespread alkaline phosphatase activity was observed in the EPC as well as in an asymmetric pattern along one lateral side of the implantation site (Figure 5B). The observed pattern of alkaline phosphatase activity in the EPC suggests an earlier onset of transgene activity in *Tpbpa-CIG* line 5, compared with the other two lines, but is more representative of the endogenous expression of *Tpbpa.* The alkaline phosphatase activity outside of the EPC appeared to extend down the side of the implantation chamber in a position where trophoblast giant cells lie (see below).

Interestingly, for Line 5, alkaline phosphatase activity was observed in a much lower proportion of implantation sites than expected under Mendelian inheritance (11.8% of the total number of conceptuses compared to the expected ratio of 25%) (Table 3). For this reason, PCR genotyping was undertaken to identify all conceptuses carrying the *Tpbpa-CIG* transgene. The presence of β-galactosidase staining was used to identify all *Z/AP* positive conceptuses. Combining these two results, it was found that 35.3% of all double transgenic conceptuses exhibited alkaline phosphatase activity. The remaining double transgenic conceptuses did not have any discernible alkalkine phosphatase activity. The ratio of double transgenic placentas observed upon PCR genotyping was within the expected values under normal Mendelian inheritance ($p>0.10$)

Figure 5. **Expression of *Tpbpa-CIG* in the developing placenta**. *Tpbpa-CIG* mice were crossed to the Cre recombinase double reporter line Z/AP (Lobe et al., 1999). hPLAP activity is present in cells that have undergone Cre-mediated recombination. (A) Expression of *Tpbpa-CIG* at various developmental stages for three independent transgenic lines. The boxes at E8.5 correspond to higher magnification images in Figure 5B. (B) The three *Tpbpa-CIG* transgenic lines demonstrate different levels of activity at E8.5. Lines 3 and 4 have a small number of hPLAP positive cells in the EPC (arrowheads). Line 5 exhibits a more widespread pattern of expression in the EPC and asymmetrically along one lateral edge of the implantation site. The right hand panel is a higher magnification view, showing staining in TGCs but not in neighboring diploid cells. (C) Cre recombinase expression was not detected in secondary trophoblast giant cells in all three transgenic lines. Asterisks indicate secondary trophoblast giant cells. Dec, decidua; epc, ectoplacental cone; Lab, labyrinth; SpT, spongiotrophoblast; TGC, trophoblast giant cells.

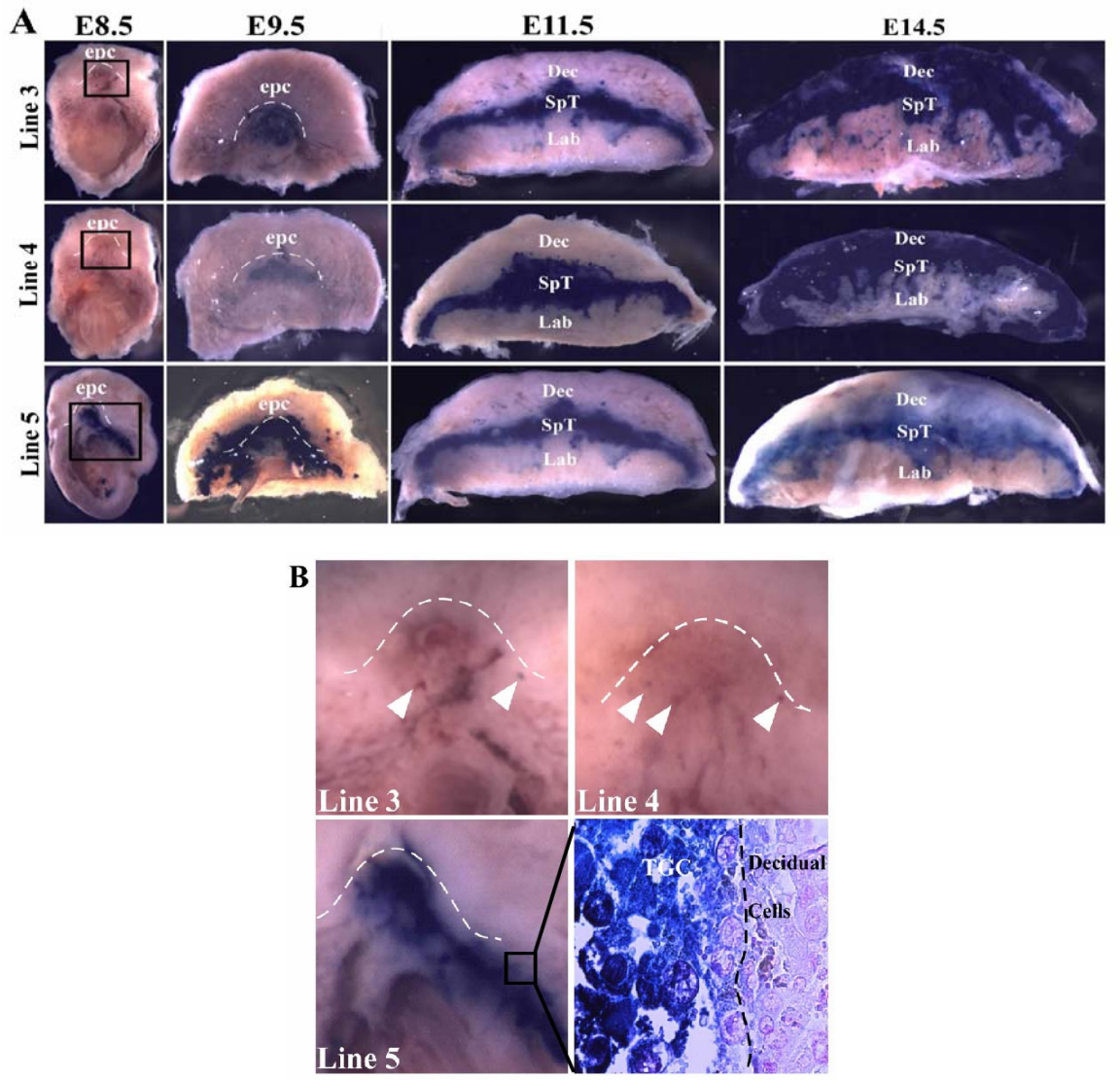

A
E8.5
E9.5
E11.5
E14.5
Line 3
Line 4
Line 5
epc
Dec
SpT
Lab
B
Line 3
Line 4
Line 5
TGC
Decidual
Cells
C
Line 3
Line 5
Tpbpa
Cre
4X
20X

Table 3. Inheritance of the *Tpbpa-CIG* transgene based on the presence of hPLAP staining in the EPC or placenta in Tpbpa-CIG x Z/AP crosses

Stage		Line 3	Line 4	Line 5
E8.5	Number	21	12	37
	hPLAP positive (%)	5 (23.8%)	3 (25.0%)	4 (10.8%)
E9.5	Number	24	19	22
	hPLAP positive (%)	6 (25.0%)	8 (42.1%)	10 (45.5%)
E11.5	Number	25	11	21
	hPLAP positive (%)	4 (16.0%)	2 (18.2%)	5 (23.8%)
E14.5	Number	18	24	25
	hPLAP positive (%)	7 (38.9%)	11 (45.8%)	8 (32.0%)
Total	Number	88	66	105
	hPLAP positive (%)	22 (25.0%)	21 (31.8%)	27 (25.7%)

Number, the total number of EPCs or placentas that were stained for transgene activity; hPLAP positive, the number of EPCs or placentas with detectable hPLAP staining indicating Cre excision has occurred in a mouse carrying the Z/AP transgene.

pattern more closely resembled that observed in the other two transgenic lines (Figure 5A). Alkaline phosphatase expression appeared to be restricted to the spongiotrophoblast region of the placenta at E11.5 and at E14.5, as for the endogenous expression of *Tpbpa* mRNA.

2.3.5 Confirmation of recombination with a different target transgene

Confirmation of the activity patterns observed with the Z/AP reporter line was attempted using the single reporter line, Gt Rosa Sor ^{tm1sor} (Soriano, 1999). However, this approach was problematic, as placental tissues had to be stained in β-galactosidase stain for up to 14 days in order to observe staining. After prolonged staining, though, the pattern of activity observed in *Tpbpa-CIG* and *Gt Rosa Sor* ^{tm1sor} double transgenic placentas at E11.5 and E14.5 were identical to those observed with the Z/AP reporter line (data not shown). The ectopic activity in embryos from Line 4 was also observed using the Gt Rosa Sor ^{tm1sor} reporter (Figure 4D), and expression was not observed in embryos from Lines 3 and 5. These results indicate that the Cre recombinase expressed in *Tpbpa-CIG* transgenic mice is capable of mediating recombination of at least 2 distinct target transgenes.

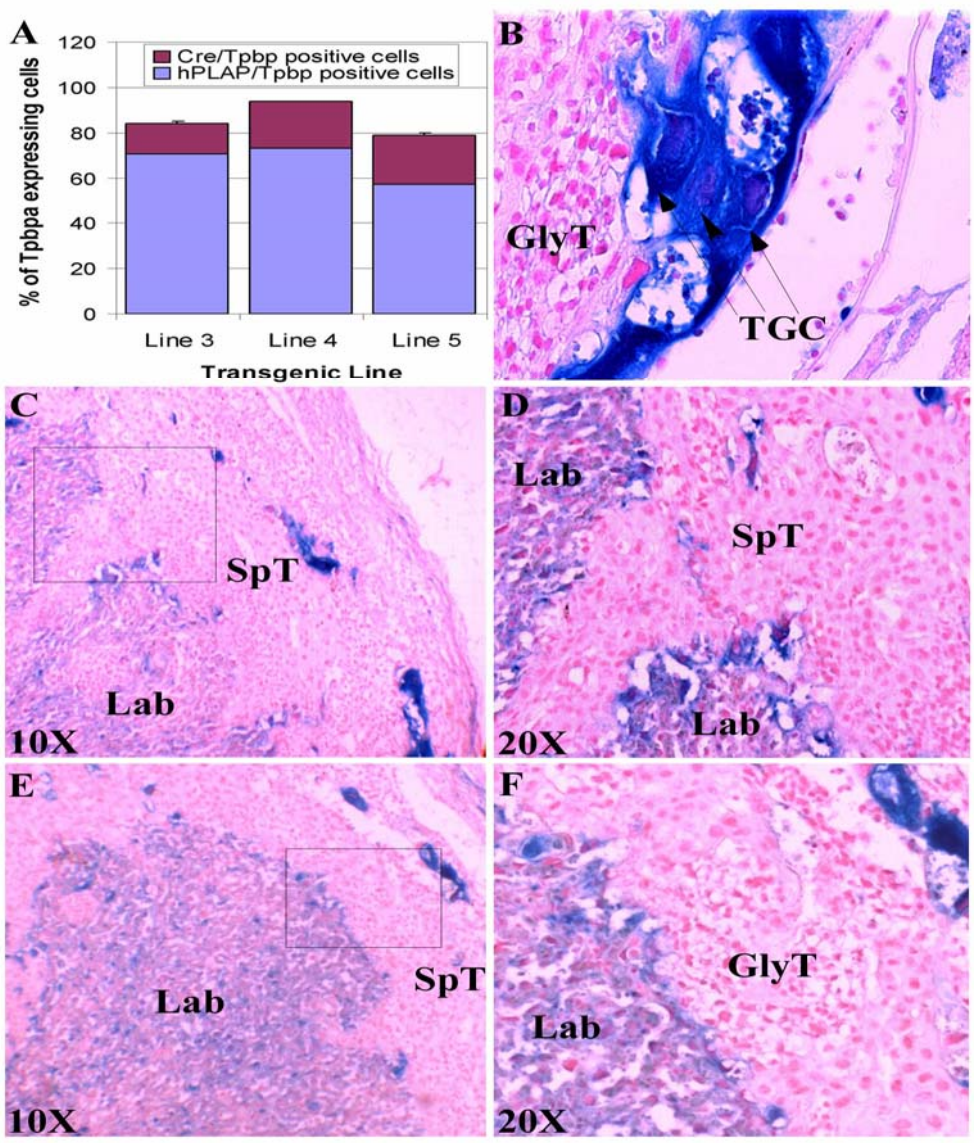
2.3.6 Assessment of recombination activity at the cellular level

To assess whether recombination occurred in all cells within the spongio-trophoblast and glycogen trophoblast cell populations, serial sections of double transgenic population, placentas at E14.5 were subjected to in situ hybridization to identify cells expressing the mRNA for *Tpbpa* (to mark the endogenously expressing population) (Lescisin et al., 1988), *Cre recombinase* and *Plf* (a trophoblast giant cell marker) (Carney et al., 1993; Linzer et al., 1985). A further slide was subjected to immunohistochemistry to identify cells expressing the human placental alkaline phosphatase (hPLAP) protein. Using these sets of slides, recombination was quantified by comparing the number of cells expressing *Tpbpa* to the number of cells co-expressing *Tpbpa* and hPLAP for each transgenic line (Figure 6). Gross examination of the slides indicated that the hPLAP antibody, which recognizes the membrane-bound enzyme, did not label glycogen trophoblast cells very well due to their morphology. These cells tend to cluster in groups of cells with a foamy appearance due to the loss of the cell granule

contents during histological processing. These clusters were always labeled strongly along the outside edges of each cluster, but cells within each cluster did not always label well. To adjust for this under-representation, the total number of cells co-expressing *Tpbpa* and *Cre* but not expressing hPLAP, were added to the total cell number expressing *Tpbpa* and hPLAP (Figure 6). The proportion of *Tpbpa*-expressing cells that also expressed either hPLAP or *Cre* was variable between lines (84.2% of cells in Line 3 (n=833), 93.6% of cells in Line 4 (n=537) and 78.7% of cells in Line 5 (n=371)) but these differences were not statistically significant. Based on morphology and on cell markers, all three *Tpbpa-CIG* transgenic lines appeared to go recombination in most, if not all, of the cells that express the endogenous *Tpbpa* gene.

The failure to detect hPLAP and/or Cre expression in 100% of spongiotrophoblast cells suggested possible mosaic expression or activity of the *Tpbpa-CIG* transgene. However, it was also possible that the analysis under-represented actual recombination events because expression of hPLAP may lag even if recombination has occurred. Therefore, a parallel approach was undertaken to quantify the recombination events more precisely. Placentas from all three transgenic lines were stained for β-galactosidase activity, paraffin-embedded, sectioned and counterstained with nuclear fast red. These sections were examined to determine if any cells that morphologically resembled spongiotrophoblast or glycogen trophoblast cells had failed to undergo recombination as indicated by continued expression of β-galactosidase. Examination of double transgenic placentas at E14.5 revealed that for all three transgenic lines, recombination had taken place in all cells known to express endogenous *Tpbpa* (Figure 6 and data not shown). The number of double transgenic placentas examined was 7 from Line 3, 11 from Line 4 and 8 from Line 5. Each of these placentas was represented by a minimum of 4 sections which were examined for mosaic recombination. Cells that continued to express β-galactosidase included the syncytiotrophoblast of the labyrinth and some trophoblast giant cells.

Figure 6. **Cre-mediated recombination occurs in all spongiotrophoblast and glycogen trophoblast cells by E14.5.** (A) Serial analysis of cell-type specific markers reveals that >75% of cells expressing *Tpbpa* also express hPLAP, the indicator of recombination, or Cre recombinase (n=1731). There is no significant difference between the three transgenic lines. (B-F) Histological sections through lacZ stained placentas. Positive lacZ staining indicates the absence of Cre-mediated recombination. (B) Trophoblast giant cells are strongly positive for b-galactosidase expression but neighboring glycogen trophoblast cells are negative. (C-D) Syncytiotrophoblast cells in the labyrinth have not undergone Cre-mediated recombination but neighboring spongiotrophoblast cells have. (D; higher magnification view of C) (E-F) Glycogen trophoblast cells have undergone Cre-mediated recombination (F; higher magnification view of E). GlyT, glycogen trophoblast; Lab; labyrinth; SpT, spongiotrophoblast; TGC, trophoblast giant cell.


A
% of Tpbpa expressing cells
120
100
80
60
40
20
0
Cre/Tpbp positive cells
hPLAP/Tpbp positive cells
Line 3
Line 4
Line 5
Transgenic Line
B
GlyT
TGC
C
SpT
Lab
10X
D
Lab
SpT
Lab
20X
E
Lab
SpT
10X
F
GlyT
Lab
20X

2.3.7 Cell lineage analysis of Tpbpa-expressing cells

One of the great advantages of Cre-activated reporter lines is that a recombination event in a specific cell is a permanent, heritable change that is passed on to all progeny of that cell, even if that cell no longer continues to express Cre. This feature of the system allows for lineage tracing of cells that have expressed Cre recombinase. Based on the observation that the three lines of *Tpbpa-CIG* mice undergo recombination in the majority of cells that express *Tpbpa* endogenously, these mice were used to assess the fate of cells that have expressed *Tpbpa*.

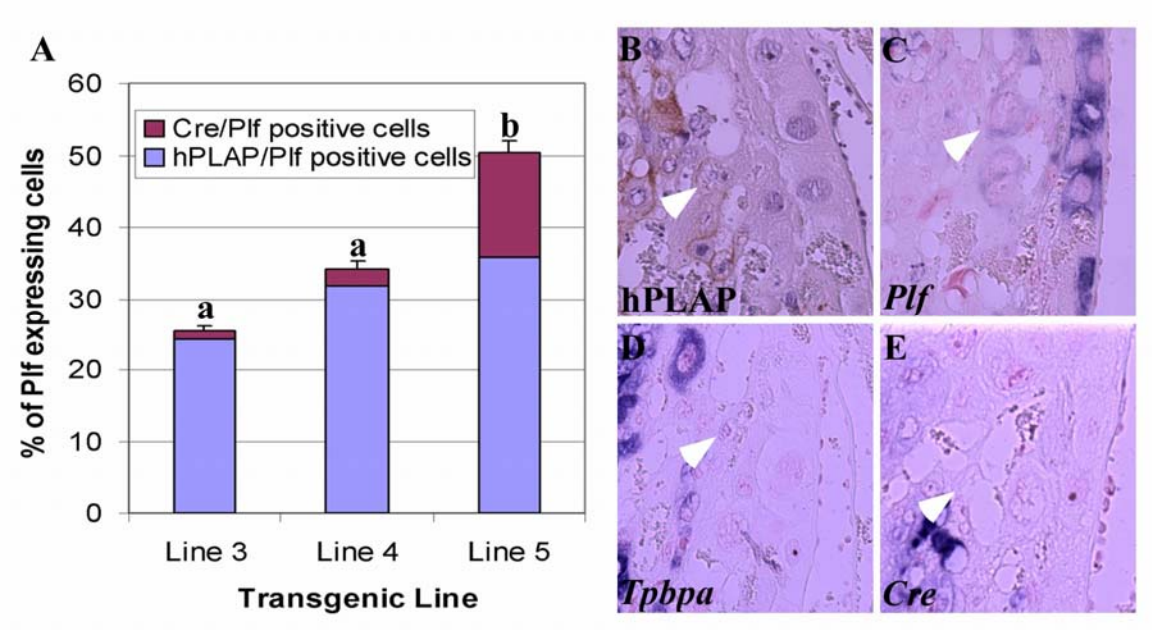
2.3.7.1 Cell fates at E8.5 using Tpbpa-CIG Line 5

The widespread and asymmetrical pattern of alkaline phosphatase activity in Line 5 at E8.5 provided an opportunity to examine cell lineage at this early stage. The location of the cells along the lateral side of the implantation site suggested that these cells represented secondary trophoblast giant cells, but the cell type could not be identified at the whole mount level. To more precisely localize this asymmetric pattern of alkaline phosphatase activity, implantation sites were carefully dissected and the orientation of each implantation site was physically marked prior to staining for alkaline phosphatase activity. One litter was examined, and of 15 implantation sites, 2 of these stained for alkaline phosphatase activity. In both cases, the asymmetric distribution of the cells was oriented toward the ovary. Histological sections showed that the alkaline phosphatase activity was indeed limited to trophoblast giant cells (Figure 5B). Importantly, *Cre* mRNA expression was not detected in secondary trophoblast giant cells at E9.5 (Figure 5C and data not shown). Trophoblast giant cells (n=149) were assessed in three different Line 5/ZAP transgenic conceptuses and Cre mRNA was never detected. Similarly, for line 3, Cre mRNA was never detected (n=485 cells; 4 double transgenics). Therefore, the alkaline phosphatase activity in the secondary giant cells in line 5 was not due to ectopic expression of *Tpbpa-CIG* in trophoblast giant cells, but rather due to Tpbpa-expressing EPC cells that subsequently differentiated into giant cells.

2.3.7.2 Cell fates at E14.5 using all three *Tpbpa-CIG* transgenic lines

In order to study the fate of *Tpbpa* expressing cells, serial sections were used to examine expression patterns of the reporter protein, hPLAP, and the mRNA expression

Figure 7. **A proportion of secondary trophoblast giant cells arise from *Tpbpa*-expressing precursor cells**. (A) Serial analysis of cell-type specific markers indicates that >25% of *Plf* positive giant cells are hPLAP positive or Cre positive (n=532 cells from 10 double transgenic placentas {4 from Line 3, 2 from Line 4 and 4 from Line 5}). Values with different superscripts are significantly different ($p<0.05$). (B-E) Representative image of a *Plf* (C) positive trophoblast giant cell that also expressed hPLAP (B), but not *Tpbpa* (D) or *Cre* (E) (arrowhead).

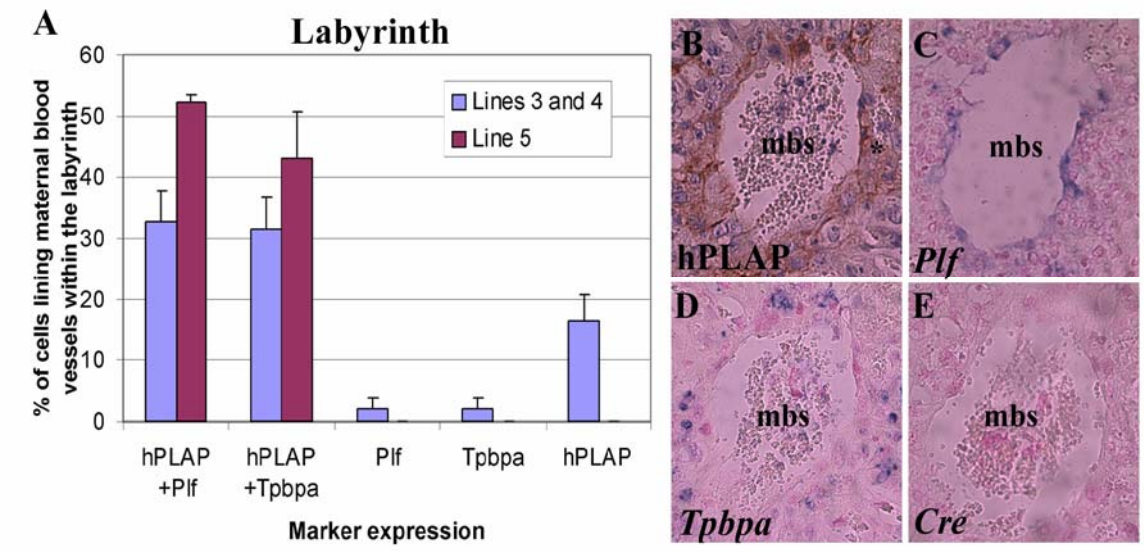

A
60
50
40
30
20
10
0
% of Plf expressing cells
Cre/Plf positive cells
hPLAP/Plf positive cells
a
a
b
Line 3
Line 4
Line 5
Transgenic Line
B
hPLAP
C
Plf
D
Tpbpa
E
Cre

patterns of *Tpbpa* (Lescisin et al., 1988), *Cre* and *Plf* (Carney et al., 1993; Linzer et al., 1985). Initial overall examination of the slides indicated that, as expected, the majority of hPLAP expressing cells also expressed *Tpbpa* and *Cre*, or both. To quantify expression patterns, random fields were selected and the markers expressed by each cell in the field were ascertained. hPLAP expressing cells were not often seen within the labyrinth layer of the placenta, except in association with large maternal blood spaces, including the central arterial canals and the peripheral venous channels. Similarly, outside the trophoblast giant cell layer, in the maternal decidua, hPLAP positive cells were only seen in association with maternal spiral arteries entering the placenta.

The proportion of *Plf* expressing trophobast giant cells that were derived from *Tpbpa*-expressing cells through secondary giant cell differentiation was found to be quite high. The cells were counted in random fields throughout the placenta, but did not include cells that were directly lining maternal blood spaces (discussed below). This number was then adjusted to include *Plf* positive cells that were also *Cre* positive, to account for cells that were expressing Cre recombinase without strong expression of the reporter (Figure 7). These results indicated that Line 5 had a significantly larger proportion of *Plf* expressing cells (50.4%, n=115 cells from 4 double transgenic placentas) that also express hPLAP or *Cre*, compared to both of the other lines (25.4 %, n= 335 cells from 4 double transgenic placentas for Line 3; 34.2%, n= 85 cells from 2 double transgenic placentas for Line 4) ($P<0.03$). This result was expected, as the onset of transgene expression is earlier for Line 5 than for the other two lines, and it would appear that more of the precursors of secondary trophoblast giant cells became labeled in the early stages of development.

As mentioned previously, hPLAP-positive cells within the labyrinth layer were found in close association with large maternal blood spaces. These blood spaces were identified as central arterial canals and large venous channels based on their locations and large size. The expression of each marker was assessed in each cell directly surrounding these large maternal blood spaces, within one cell layer. Based on the expression patterns revealed in the whole mount analysis, results for Lines 3 and 4 were grouped together and compared to results for Line 5. Smaller maternal blood sinuses located throughout the

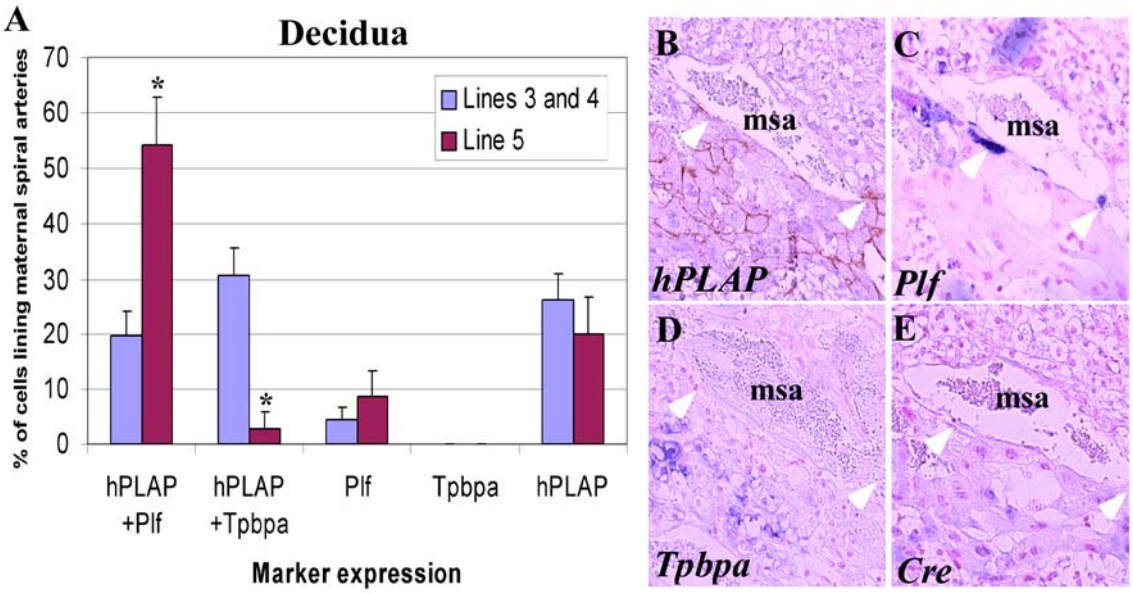
Figure 8. **Cells lining large maternal blood spaces within the labyrinth are derived from the ectoplacental cone**. (A) Serial analysis of cell-type specific markers indicates that >95% of the cells immediately lining the central arterial canals and large venous channels in the labyrinth are derived from *Tpbpa*-expressing cells in the ectoplacental cone (n=126). The asterisk indicates a statistically significant difference in the proportion of cells expressing only hPLAP, and no other marker, between Lines 3 and 4 and Line 5. (B-E) Representative images of a maternal blood space demonstrating that the majority of the cells express hPLAP (B), the marker of recombination and that these cells may also express *Plf* (C) or *Tpbpa* (D). mbs; maternal blood space.


A
Labyrinth
% of cells lining maternal blood vessels within the labyrinth
60
50
40
30
20
10
0
Lines 3 and 4
Line 5
hPLAP +Plf
hPLAP +Tpbpa
Plf
Tpbpa
hPLAP
Marker expression
B
mbs
*
hPLAP
C
mbs
Plf
D
mbs
Tpbpa
E
mbs
Cre

labyrinth region were not examined, as these were not readily identifiable in histological sections. For the large maternal canal/channel blood spaces within the labyrinth, cells were observed that expressed hPLAP plus *Plf* and hPLAP plus *Tpbpa* (Figure 8). In Lines 3 and 4 (n=98 cells from 6 double transgenic placentas), a significantly larger proportion of cells expressing only hPLAP,and no other marker (*Plf*, *Tpbpa* or *Cre*), were observed as compared to for Line 5 (n=44) ($P<0.01$).

For maternal spiral arteries within the maternal decidua, a significantly larger proportion of cells in Line 5 (54.3%, n=35 cells from 4 double transgenic placentas) expressed hPLAP and *Plf* compared to Lines 3 and 4 (19.8%, n=91 cells from 6 double transgenic placentas) ($P<0.01$) (Figure 9). These cells were located in a position such that they were directly lining the lumen of the spiral artery, and did not resemble glycogen trophoblast cells. The cells tended to have either an elongated, spindle-like shape or to be rounded, with a more dense appearance than glycogen trophoblast cells. The presence of hPLAP positive cells in this location indicates that the trophoblast cells lining the maternal spiral arteries are largely represented by trophoblast giant cells that are derived from EPC/spongiotrophoblast with cells. These cells likely differentiate during earlier stages of development, along with what is recognized as the secondary giant cell population. As described by Hemberger *et al.* (2003), the *Plf* positive cells associated with the maternal spiral arteries did not have the characteristic large size and nuclei typical of trophoblast giant cells. These cells often had an elongated, spindle like appearance, as described previously (Hemberger et al., 2003). Cells lining maternal spiral arteries in Lines 3 and 4 were significantly more likely to co-express hPLAP and *Tpbpa* than were the same population of cells in Line 5 ($P<0.01$). Both lines had a large number of cells expressing only hPLAP, and none of the other markers (*Plf, Tpbpa* or *Cre*) examined.

Figure 9. **Cells immediately lining the lumen of maternal spiral arteries are derived from the ectoplacental cone**. (A) Serial analysis of cell-type specific markers indicates that >92% of the cells immediately lining the maternal blood spaces in the labyrinth are derived from *Tpbpa*-expressing cells in the ectoplacental cone (n=91). Asterisks indicate a statistically significant difference in the proportions of cells expressing hPLAP + Plf, and hPLAP+Tpbpa between Lines 3 and 4 and Line 5. (B-E) Representative images of a maternal spiral artery showing hPLAP-positive secondary trophoblast giant cells (B-C) in association with the lumen of the artery. msa, maternal spiral artery. These cells do not express *Tpbpa* (D) or *Cre* (E).


A
Decidua
% of cells lining maternal spiral arteries
70
60
50
40
30
20
10
0
*
*
Lines 3 and 4
Line 5
hPLAP +Plf
hPLAP +Tpbpa
Plf
Tpbpa
hPLAP
Marker expression
B
msa
hPLAP
C
msa
Plf
D
msa
Tpbpa
E
msa
Cre

2.4 Discussion

This study has described the generation and characterization of transgenic mice expressing Cre recombinase and EGFP specifically in the spongiotrophoblast layer of the placenta under the control of the *Tpbpa* promoter. Three independent lines were generated, and one of these lines (Line 4) faithfully recapitulates the endogenous expression of *Tpbpa* in the placenta (Carney et al., 1993; Lescisin et al., 1988). The *Tpbpa-CIG* transgenic mice were crossed with the *Z/AP* reporter mice (Lobe et al., 1999) to generate a heritable change in all cells that initially expressed the Cre transgene. This approach allowed for cell lineage to be examined within the mouse placenta. Analysis of serial sections through E14.5 double transgenic placentas labelled for cell type specific markers as well as Cre recombinase and the reporter protein, hPLAP, identified descendents of the *Tpbpa* expressing population. The results of this study indicate that EPC and spongiotrophoblast cells can contribute to the secondary trophoblast giant cell population. Descendents of this population were also seen in association with maternal blood spaces both within the labyrinth and in the maternal decidua. It has been shown previously that cells lining the maternal blood spaces within the labyrinth of mice are trophoblast cells, not endothelial cells (Adamson et al., 2002; Wooding, 1994). The results of this study suggest that the cells lining the maternal blood spaces within the labyrinth are derived from the EPC, and may be a mixture of spongiotrophoblast and giant cells. In the maternal decidua, the majority of cells observed in association with maternal spiral arteries were *Plf* expressing trophoblast giant cells, descendent from the *Tpbpa*-expressing EPC population. This supports previous studies which have identified the endovascular invading cell type lining maternal spiral arteries as being *Plf*-expressing trophoblast giant cells(Adamson et al., 2002; Hemberger et al., 2003).

2.4.1 Expression of the Tpbpa-CIG transgene during development recapitulates endogenous placental expression of *Tpbpa*

Tpbpa-CIG and Z/AP double transgenic EPCs and placentas were stained for alkaline phosphatase activity at various developmental stages to identify the onset of Examination of alkaline phosphatase activity indicated that for Lines 3 and 4, the

transgene is expressed as early as E8.5 in a subset of cells in the EPC. This expression became more widespread throughout the EPC by E9.5, and was restricted to the spongiotrophoblast region of the mature placenta at E11.5 and E14.5. These two lines of Tpbpa-CIG mice exhibit transgene expression that resembles that of the endogenous *Tpbpa* gen. However, the onset of expression is slightly delayed compared to the endogenous gene.

The onset of expression for Line 5 appears to occur earlier than in Lines 3 and 4, based on the presence of more widespread alkaline phosphatase staining throughout the EPC at E8.5. The expression observed, is more representative of the endogenous *Tpbpa* expression pattern. In addition, the expression pattern exhibited by the double transgenic mice is asymmetrical, with alkaline phosphatase expression extending beyond the EPC and along one side of the implantation site. The expression of the transgene is also more widespread at E9.5 in Line 5 compared to the other two transgenic lines, which is likely a direct result of the more widespread expression observed at E8.5. At E11.5 and E14.5, the expression of the transgene in Line 5 resembles the pattern observed for the other two transgenic lines.

The identification of cells expressing the transgene was hampered by the use of whole-mount staining to study the onset and pattern of expression. At the whole mount level, expression can only be attributed to regions of the placenta and not to specific cell types. In particular, expression in the trophoblast giant cell population cannot be directly identified, because of the small number and disperse localization of these cells.

2.4.2 The ROSA26 locus is not strongly expressed in the spongiotrophoblast

In an attempt to confirm the expression patterns observed in crossing the Tpbpa-CIG transgenic lines to the Z/AP double reporter (Lobe et al., 1999), these lines were crossed to the single reporter strain, Gt Rosa Sor ^{tm1sor} (Soriano, 1999). This line of reporter mice contains a loxP-flanked stop sequence, followed by a region coding for β-galactosidase. The construct was targeted to the ROSA26 locus, which had previously been identified in a gene trap screen as being constitutively active during development (Friedrich and Soriano, 1991; Zambrowicz et al., 1997). This approach proved

problematic, as the placentas had to be left in β-galactosidase stain for very long periods, up to 14 days, to visualize β-galactosidase staining in the spongiotrophoblast region. The requirement for extended staining periods suggests that the ROSA26 locus may not be strongly expressed in the spongiotrophoblast region of the placenta. Intriguingly, similar results were observed when ROSA26 ES cells in which the β-galactosidase gene was knocked in to the ROSA26 locus were used in ES cell ↔ tetraploid aggregation experiments (Tanaka et al., 1997). When tetraploid ROSA26 cells are aggregated with wild type ES cells, the tetraploid cells will only contribute to the trophoblast lineages and the primitive ectoderm (Nagy et al., 1990; Tanaka et al., 2001). LacZ staining of the resultant placentas demonstrated that β-galactosidase activity was only observed in the labyrinthine trophoblast and spongiotrophoblast cell types, and not in the non-trophoblast lineages (Tanaka et al., 1997). The staining observed in the placenta is much stronger in the labyrinth as compared to the spongiotrophoblast region, supporting the idea that the ROSA26 locus is not strongly expressed in these cell types.

2.4.3 The Tpbpa-CIG transgene is expressed in all spongiotrophoblast and glycogen trophoblast cells

Two methods were undertaken to assess the potential for mosaic expression of the transgenes within the spongiotrophoblast and glycogen trophoblast cells at E14.5. First, halves of placentas carrying both the Tpbpa-CIG transgene and the Z/AP transgene were stained for β-galactosidase activity to identify cells in which recombination had not occurred. These placentas were then paraffin-embedded and sectioned to allow for analysis at the cellular level. If mosaic expression or recombination was occurring, one would expect to see patches of blue cells that had not undergone excision within the spongiotrophoblast region of the placenta. Examination of these sections revealed that all cells identified as spongiotrophoblast or glycogen trophoblast cells based on location and morphology did not express β-galactosidase, indicating that Cre-mediated excision had occurred in all of these cells.

A second method to assess mosaicism involved the use of cell-type specific markers, which were used to label the cells by in situ hybridization. Serial sections were

analyzed for the expression of cell type specific markers. The total number of cells expressing *Tpbpa* was compared to the number of cells expressing hPLAP and *Tpbpa* for all three transgenic lines. Based on the observation that glycogen trophoblast cells do not label well with the hPLAP antibody, the cells expressing *Tpbpa* and Cre recombinase were added to the hPLAP and *Tpbpa*-expressing cells to adjust for the under-representation of this population that was likely to occur. These results indicated that >78% of *Tpbpa*-expressing cells either expressed *Cre* or had undergone Cre-mediated excision.

The loss of β-galactosidase activity is likely a more reliable indicator of excision, due to the stability of the β-galactosidase protein. This protein is highly stable, and its half life has been estimated by histochemical methods to be approximately 24 hours (Smith et al., 1995). Given that β-galactosidase activity was not observed in any cells identified by morphology and location within the placenta as being spongiotrophoblast or glycogen trophoblast cells, it is likely that excision had occurred in all cells at least 24 hours prior to collection of the tissue samples. Additionally, given that hPLAP is a membrane-bound isoform of the protein (Ogata et al., 1988), labelling of cells was assessed based on complete or near-complete staining of the cell membrane by hPLAP. This approach resulted in the exclusion of cells with membranes that were not completely stained for hPLAP, and may have undergone Cre-mediated excision more recently. Based on these points, the absence of β-galactosidase activity is likely to represent a more accurate indicator of Cre-mediated excision in this particular experiment. These results suggest that Cre-mediated excision has occurred in all cells of the spongiotrophoblast and glycogen trophoblast populations for all three transgenic lines.

2.4.4 *Tpbpa*-expressing cells do not contribute to the syncytiotrophoblast of the labyrinth

Analysis of the serial sections described in Section 4.6 revealed that hPLAP positive cells were not seen in the labyrinth, except in association with maternal blood spaces (discussed below). Cells expressing only hPLAP and none of the other markers were only observed rarely. These results indicate that cells derived from *Tpbpa*-

expressing progenitors do not make a significant contribution to the labyrinth. This raises the question of what precursor population gives rise to the syncytiotrophoblast cells in the labyrinth, as these cells do not arise from the same population as the spongiotrophoblast, glycogen trophoblast and trophoblast giant cells. The precursor population from which the syncytiotrophoblast lineage arises has yet to be identified, but is likely found within the chorion. The expression of certain syncytiotrophoblast markers begins within the chorion prior to syncytiotrophoblast differentiation (Cross, 2000), which strongly implicate these cells in labyrinth formation. Cell lineage tracing of cells derived from the chorion may provide further evidence for this relationship.

2.4.5 Spongiotrophoblast cells and secondary trophoblast giant cells share a common precursor population

The earlier onset of transgene expression in Line 5 allowed for the examination of early cell lineage. The asymmetrical pattern of alkaline phosphatase staining that was observed at E8.5 suggested that the cells along the lateral edge of the implantation site were descendent from the *Tpbpa*-expressing population in the EPC. Secondary trophoblast giant cell differentiation can be detected as early as E6.5 (Carney et al., 1993), and based on the location of these cells it seemed likely that the positively staining cells belonged to this population. These results may indicate that secondary trophoblast giant cells and *Tpbpa*-expressing spongiotrophoblast and glycogen trophoblast cells share a common precursor population.

To further study cell lineage, serial marker analysis was conducted at E14.5 in all three transgenic lines. To assess the contribution of *Tpbpa*-expressing cells to the secondary trophoblast giant cell population, the percentage of cells expressing hPLAP in the total population of cells expressing the trophoblast giant cell marker, *Plf*, was determined for each of the transgenic lines. To this was added the cells also expressing both *Cre* and *Plf*, to account for any possible under-representation of the hPLAP expressing population, as described previously. The adjusted percentage of *Plf* expressing cells that express either hPLAP or *Cre* was significantly higher in Line 5 than in the other two lines. This is not surprising, given that the onset of expression was

earlier and more widespread in Line 5 than in the other two lines. This would result in more proliferating cells undergoing Cre-mediated excision, and therefore more cells would ultimately express hPLAP. Greater than 20% of the *Plf* expressing trophoblast giant cells were also hPLAP positive in all three transgenic lines, indicating that at least some portion of cells that begin to express *Tpbpa* can then turn off the expression of this marker and differentiate to form trophoblast giant cells *in vivo*.

Secondary trophoblast giant cells differentiating from the outer edge of the EPC are detectable as early as E6.5 (Carney et al., 1993). The EPC also gives rise to the spongiotrophoblast in the mature placenta (Cross et al., 1994). Indirect evidence that trophoblast giant cells and spongiotrophoblast cells share a common precursor population has been demonstrated by explant culture of extraembryonic ectoderm and EPC cells (Carney et al., 1993). When extraembryonic ectoderm cells were explanted and placed in culture, they passed through an intermediate stage where they expressed *Tpbpa* prior to differentiating to cells that both expressed the markers of and were morphologically identifiable as trophoblast giant cells. When EPC cells were explanted and maintained in culture, the onset of *Tpbpa* expression was very early, and these cells differentiated to form trophoblast giant cells at a rate that was accelerated compared to extraembryonic ectoderm cultures. The fact that both extraembryonic ectoderm explant and EPC explant cultures expressed the spongiotrophoblast marker *Tpbpa* prior to differentiating to generate trophoblast giant cells supports the proposal that these two cell types share a common precursor population. This suggestion is now supported by *in vivo* evidence that cells expressing *Tpbpa*, a marker typical of spongiotrophoblast, are capable of differentiating to form secondary trophoblast giant cells.

It is intriguing, however, that greater than half of the trophoblast giant cells *in vivo* are not derived from cells that have expressed *Tpbpa*. Previous studies using trophoblast stem cell cultures have demonstrated that retinoic acid is capable of blocking spongiotrophoblast formation and promoting trophoblast giant cell differentiation *in vitro* (Yan et al., 2001). In the presence of high levels of retinoic acid, trophoblast stem cells did not express detectable levels of *Tpbpa*, even after only one day of differentiation, and at levels of retinoic acid that were only partially inhibitory of spongiotrophoblast fate,

Tpbpa and another spongiotrophoblast marker, *Cea4* were still detected although at lower levels than in the absence of retinoic acid. Based on these observations, Yan *et al*. (2001) proposed a model whereby trophoblast stem cells first differentiate to a bipotent precursor cell that may then form either trophoblast giant cells or spongiotrophoblast cells. The idea that trophoblast giant cells may directly differentiate from a progenitor cell is not entirely improbable; mural trophectoderm cells do not express *Tpbpa* prior to forming primary trophoblast giant cells. It is therefore possible that some of the cells in the ectoplacental cone are a bipotent precursor population that can differentiate to give rise to spongiotrophoblast or trophoblast giant cells.

2.4.6 The cells lining maternal blood spaces are predominantly secondary trophoblast giant cells

One of the observations made during this study was that trophoblast giant cells that arose from the differentiation of cells that had previously expressed *Tpbpa* were often associated with maternal blood. This association was seen both within the labyrinth, where these cells were observed lining large maternal blood spaces, as well as in the decidua where these cells were observed lining the maternal spiral arteries.

Maternal blood spaces within the labyrinth are lined by trophoblast cells rather than the endothelial cells that are typical in most mammals; this type of placenta is referred to as ‘hemochorial’ (Wooding, 1994). The origin of these cells lining the maternal blood spaces has remained unclear, however the arrangement of these cells is highly ordered. The smaller maternal sinusoid spaces in the labyrinth are partitioned from each other by three cell layers; the innermost layer lining the lumen has been described as cuboidal mononuclear cells, while the two outer layers are composed of multinucleated syncytiotrophoblast cells (Adamson et al., 2002). These small maternal sinusoid spaces could not readily be identified on histological sections and were therefore not included in the quantification. Previous studies have indicated that the maternal sinusoid blood spaces in the labyrinth are only readily observed in histological sections when the maternal arterial flow is ligated before fixation, as these small blood spaces collapse

during fixation (Adamson et al., 2002). As this procedure was not followed for this study, the maternal sinusoid spaces were not readily identifiable.

In this study, greater than 80% of the cells directly lining the lumen of the large maternal blood spaces in the labyrinth at E14.5 were hPLAP positive in Lines 3 and 4, which were combined due to their similar transgene expression patterns. For Line 5, virtually all (95.5%) of the cells directly lining the maternal blood spaces at E14.5 were hPLAP positive. Again, this difference probably arises from the earlier onset of transgene expression in these lines, resulting in a larger population of labelled cells. These hPLAP positive cells also expressed either *Plf* or *Tpbpa*. For Line 5, these were the only expression patterns observed. In Lines 3 and 4, some cells were observed that expressed only one of hPLAP, *Tpbpa* or *Plf*.

These observations suggest that cells derived from the EPC become distributed throughout the labyrinth region of the placenta as part of the rearrangement of trophoblast cells that occurs to shape the maternal blood spaces. These cells line the maternal blood spaces in the central arterial canal and the venous channels, and are a mixture of trophoblast giant cells and spongiotrophoblast cells. Due to the small numbers observed in this study, a more detailed examination of this cell lineage question should be undertaken to confirm the results. The presence of cells derived from the EPC within the labyrinth supports the suggestion that spongiotrophoblast morphogenesis is important for the proper development of the labyrinth layer. The rearrangement of EPC-derived trophoblast cells to generate maternal blood spaces within the labyrinth may be involved in establishing the pattern of fetal blood flow into the labyrinth, by physically restricting the domains into which fetal blood vessels can grow.

In this study, greater than 75% of the trophoblast cells directly lining the lumen of maternal spiral arteries within the maternal decidua were positive for hPLAP expression in all three lines, indicating that they had at one point expressed *Tpbpa*. A proportion of these cells also expressed the trophoblast giant cell marker, *Plf*. In Lines 3 and 4, which were grouped together based on their similar transgene expression pattern, this proportion was significantly lower than that observed for Line 5. This difference is likely due to the earlier onset of transgene expression in Line 5, resulting in a larger population of cells

that have undergone excision. A small proportion of cells (<5%) in all three lines were also positive for *Plf* alone, although this may simply be an artefact of the method as these cells may not have been included in the hPLAP expressing population due to the stringent inclusion of only cells that were essentially completely bound by hPLAP expression. Alternately, the cells may not have been perfectly correlated on the serial sections from one slide to the next, resulting in slight differences in marker assignment between slides.

The *Plf* expressing cells lining maternal spiral arteries were not readily identifiable as trophoblast giant cells. Rather than the large cell and nuclear size characteristic of this cell type, these cells were elongated and had smaller nuclei. Interestingly, a significantly higher proportion of the hPLAP positive cells lining the maternal spiral arteries in Lines 3 and 4 were also *Tpbpa* positive. Line 5 had very few cells demonstrating the expression of these two markers together.

Previous studies have shown that trophoblast giant cells line the spiral arteries within the maternal decidua region of the placenta as early as E8.5 (Hemberger et al., 2003). This association occurred only within 150-300 μm from the main trophoblast giant cell layer, and the trophoblast giant cells were only seen closely associated to the spiral arteries. Based on this observation, it was suggested that trophoblast giant cells invade into the maternal spiral arteries by an endovascular route (Adamson et al., 2002). A more detailed study of the endovascular invasion described invasion of trophoblast giant cells into the maternal spiral arteries as early as E8.5 (Hemberger et al., 2003). These cells were shown to interact with and replace the endothelial lining of the spiral artery; proximal to the main trophoblast giant layer spiral arteries were almost completely enclosed by *Plf* positive cells while further away fewer *Plf* positive cells were observed with an increase in endothelial cells. Of note, the *Plf* positive trophoblast giant cells did not have the typical large nucleus and cell size usually associated with this cell type, but rather had a spindle-like appearance and smaller nuclei (Hemberger et al., 2003). This is in agreement with the morphology observed in this study.

Given that endovascular trophoblast giant cell invasion of the maternal spiral arteries has occurred by E8.5, these cells likely arise from the secondary giant cell population that begins to differentiate by E6.5 (Carney et al., 1993). The results of this

study, as well as previous studies (Adamson et al., 2002; Hemberger et al., 2003), suggest that trophoblast giant cells at the tip of the EPC invade into the maternal spiral arteries as they enter the EPC, replacing the endothelial lining. The loss of this endothelial lining results in a loss of maternal control over vasoconstriction and therefore increased blood flow to the implantation site (Hemberger et al., 2003). Trophoblast giant cells also produce and release several angiogenic factors, such as VEGF (vascular endothelial growth factor) and Plf, which are believed to promote the growth of new vessels into the implantation site (Adamson et al., 2002). These results suggest that secondary trophoblast giant cells that have differentiated from the EPC, invade into the maternal spiral arteries where they line the arteries, increasing blood flow to the implantation site through the loss of endothelial lining.

The actual route of invasion by trophoblast giant cells into the maternal spiral arteries is unclear based on the results of this study. Another form of invasive cell is observed in the maternal decidua beginning at E12.5, when glycogen trophoblast cells begin to invade into the decidua in an interstitial pattern (Adamson et al., 2002). It is possible that cells lining the maternal arteries at E14.5 invade into the decidua interstitially first, with the glycogen trophoblast cells and then move into the spiral arteries, displacing the endothelial lining. However, two observations suggest that this is not the case. First, previous work demonstrated that secondary trophoblast giant cells are associated with maternal spiral arteries as early as E8.5, and have already invaded to a depth as great as 300 μm (Hemberger et al., 2003). Interstitial invasion of glycogen trophoblast cells is not observed until E12.5, after endovascular invasion seems to be complete. Also, cells expressing trophoblast giant cell markers have not been seen in the decidua, except in association with the maternal spiral arteries (Adamson et al., 2002; Hemberger et al., 2003). Second, the results of this study show that for Line 5, a greater proportion of the cells lining the maternal spiral arteries are expressing both the lineage marker hPLAP and the trophoblast giant cell marker when compared to the same population in Lines 3 and 4. If the endovascular invading cells first followed a route of interstitial invasion, these would all have passed through a ‘glycogen trophoblast-like’ stage, during which *Tpbpa* would be expressed and these cells would have undergone

Cre-mediated recombination. If this were the case, we would not expect to see a difference between the lines with respect to the proportions of trophoblast giant cells that had undergone recombination. Based on these two lines of evidence, it is unlikely that endovascular cells invade interstitially prior to invading into the maternal spiral arteries, but rather they invade in a solely endovascular pattern.

CHAPTER THREE:

FUTURE DIRECTIONS

3.1 Future Direction 1: Cell Lineage Tracing at Earlier Developmental Stages

Detailed analysis of cell lineage at E14.5 revealed that *Tpbpa*-expressing cells within the EPC can contribute to the secondary trophoblast giant cell population, and that these cells are involved in the process of endovascular invasion of the maternal spiral arteries. As a mature placenta has formed by E10.5, and is completely vascularized by this point (Adamson et al., 2002; Cross et al., 2002b), it is clear that endovascular invasion occurs much earlier in development than we have examined. Previous work has identified trophoblast giant cells associated with maternal spiral arteries as early as E8.5 (Hemberger et al., 2003). Detailed analysis of cell lineage prior to the establishment of a mature placenta, using the same methods described in this study, may allow for a clearer picture of endovascular invasion to be determined. If the endovascular invading cells invade directly into the maternal spiral arteries from the tip of the EPC, one would expect to see hPLAP and *Plf* co-expressing cells in the maternal decidua only in association with the spiral arteries. If the endovascular trophoblast cells invade interstitially prior to interacting with the maternal spiral arteries, one would expect to see cells expressing hPLAP in the decidua in locations other than in strict association with the maternal spiral arteries. These cells would not be expected to express the trophoblast giant cell marker at this point, as markers for this cell type have not been detected in the interstitial regions of the decidua.

Another question which may be addressed with cell lineage tracing at earlier stages is the manner in which differentiation to form secondary trophoblast giant cells proceeds. In the present study, it was apparent that some of the cells expressing *Tpbpa* differentiate to and form trophoblast giant cells and in doing so turn off *Tpbpa* expression. This pattern of gene expression has been observed in explant cultures of extraembryonic ectoderm (Carney et al., 1993) and trophoblast stem cell cultures (Tanaka et al., 1998), where differentiating cells passed through a stage where they expressed *Tpbpa* prior to trophoblast giant cell differentiation. Also, occasional cells were observed that expressed both *Tpbpa* and *Plf*, or *Cre* and *Plf*. These cells were very rare, and are not likely representative of a normal expression pattern. *Plf* is one of the earliest markers of

trophoblast giant cell differentiation (Carney et al., 1993), and so it is possible that expression is downregulated coincident with giant cell differentiation. This could account for a small number of cells that express *Tpbpa* and *Plf*, if these represent the earliest stage in differentiation. If this is the case, *Tpbpa* and *Plf* co-expressing cells would be more common during the early stages of secondary trophoblast giant cell differentiation. We are currently examining the question of cell lineage at E9.5 using the same approach described in this study, to address the questions outlined in this section.

3.2 Future Direction 2: Lineage Tracing of Trophoblast Stem Cells *in vivo*

Given that trophoblast stem cells have not been isolated from EPC cells (Uy et al., 2002) it was not surprising to find that *Tpbpa*-expressing cells do not contribute significantly to the labyrinth layer. However, trophoblast stem cells have been isolated from the extraembryonic ectoderm (Tanaka et al., 1998; Uy et al., 2002) and from its derivative, the chorionic ectoderm, as late as the 9 somite pair stage (approximately E8.0-8.5) (Uy et al., 2002). These observations would suggest that the major proliferative population of the developing placenta resides within this tissue. However, *in vivo* evidence for this is lacking. Additionally, when extraembryonic ectoderm cells are placed in culture, these cells pass through an EPC-like stage prior to differentiating to form trophoblast giant cells (Carney et al., 1993). Although it seems unlikely that extraembryonic ectoderm contributes to the trophoblast giant cell population *in vivo*, it has been suggested that the ability of trophoblast stem cells to form trophoblast giant cells throughout pregnancy may represent a mechanism to ensure that an adequate level of hormone production is maintained by this cell type (Carney et al., 1993).

A similar approach to lineage tracing as was undertaken in this study could be used to address the question of trophoblast stem cell proliferation. This would require the generation of mice in which Cre recombinase is expressed specifically within the extraembryonic ectoderm/chorionic ectoderm population. In order to generate such mice, a promoter region driving specific expression only to these tissues, or at least exclusively to these tissues in the extraembryonic compartment, would be required. Although many genes are known to be expressed within the extraembryonic ectoderm and chorion, few of these are restricted to this compartment or expressed throughout the

compartment. There is one gene that meets these requirements, the orphan nuclear receptor *Esrrb* (estrogen-related receptor beta). *Esrrb* is expressed in a subset of cells of the extraembryonic ectoderm by E5.5 and this expression becomes more widespread throughout the extraembryonic ectoderm by E6.0 (Luo et al., 1997). As development proceeds, expression remains limited to the ectodermal regions which form the chorion at E6.5. Expression of *Esrrb* is detectable throughout the chorion at E7.5, but this expression decreases as chorioallantoic attachment occurs, and by E8.5 expression of *Esrrb* is only observed at the lateral edges of the chorion that are not in contact with the allantois (Luo et al., 1997). *Esrrb* is also expressed in undifferentiated trophoblast stem cells *in vitro* (Tanaka et al., 1998). Expression of *Esrrb* has also been observed in the primordial germ cells of the developing embryo from E11.5 to E14.5 in females and E15.5 in males, at which point the expression diminishes (Mitsunaga et al., 2004). Postnatally, expression was observed in spermatogonia at postnatal day 7 (Mitsunaga et al., 2004).

Expression of *Esrrb* in the embryo should not impede the use of the regulatory regions of this gene to study cell lineage of the extraembryonic ectoderm and chorion, as the expression in the primordial germ cells begins after the expression in the extraembryonic tissues has already disappeared. Additionally, cells in the developing embryo would not affect the outcome of cell lineage tracing questions in the extraembryonic tissues. However, if the mice were to be used for the conditional alteration of the genome, the other sites of expression may become significant.

There is one major shortcoming to the suggested use of the *Esrrb* promoter region to drive Cre expression in transgenic mice, as was done in this study. Although the chromosomal location of the gene has been mapped (Sladek et al., 1997), little work has been done to characterize the regulatory regions controlling the expression of *Esrrb*. These regulatory regions would have to be identified, and tested for their ability to direct expression in transgenic mice, which would require a great deal of work.

An alternative approach would be to insert the Cre recombinase coding region into the *Esrrb* locus using homologous recombination in ES cells (Doetschman et al., 1987). This would generate mice in which the Cre recombinase is under the direct control of the

endogenous regulatory elements of *Esrrb*. Stable cell lines that have undergone the desired homologous recombination reaction could then be used to generate chimaeric mice by ES cell/embryo aggregation (Tanaka et al., 2001; Wood et al., 1993). If this approach were chosen, the targeted locus would likely have to be maintained in a heterozygous state. This is because the *Esrrb* gene would not be expressed from the targeted allele, and embryos homozygous for a targeted disruption of the *Esrrb* died by E10.5 due to placental defects (Luo et al., 1997). Once the Esrrb-Cre mice were generated, full characterization of the transgene expression would have to be carried out to validate the expression pattern prior to using the mice for lineage tracing experiments.

Cell lineage tracing experiments such as the ones described in this study could then be undertaken to examine the contribution of *Esrrb* expressing cells to the different layers of the mature placenta. The localization of a reporter to different cell types would identify the *in vivo* ability of extraembryonic ectoderm and later the chorion, to contribute to the labyrinth, the spongiotrophoblast and the trophoblast giant cell layer, to verify the *in vitro* studies that have been done.

3.3 Future Direction 3: Applications of Tpbpa-CIG mice

The characterization of the Tpbpa-CIG transgenic mice revealed that one of the three lines recapitulates the normal expression of the *Tpbpa* gene in the developing placenta. The transgene is expressed in diploid precursor cells in the EPC by E8.5, and by E11.5 is restricted to the spongiotrophoblast layer of the mature placenta. The confirmation that the transgene follows this pattern of expression indicates that these mice will be useful in the study of placental defects, by allowing for the generation of spongiotrophoblast-specific genome alterations. Many genes have been identified that result in embryonic lethal phenotypes when knocked out, often as a result of placental defects. A few specific examples of these will be discussed here, in the context of utilizing the Tpbpa-CIG to further examine the phenotypes observed in knockout animals.

Cited1 is a transcriptional cofactor that is expressed in all the trophoblast derivates in the placenta (Rodriguez et al., 2004). The Cited1 protein is largely located in the nucleus of spongiotrophoblast and trophoblast giant cells, but is located through the cell

in both the cuboidal cells and syncytiotrophoblast of the labyrinth. *Cited1* was knocked out in all cell types using Cre-loxP technology (Rodriguez et al., 2004). Examination of the resultant conceptuses revealed that placental organization was disrupted in *Cited1*-null animals (Rodriguez et al., 2004). Notably, there was an increase in the spongiotrophoblast layer with a concomitant decrease in the labyrinth layer. The organization of the spongiotrophoblast was irregular, with larger projections into the labyrinth. The labyrinth layer demonstrated defects in maternal blood spaces; the maternal sinusoids were less branched and had a larger diameter than wild type controls, and the result was a decreased surface for exchange with fetal vessels due to the discrepancy in size between the two blood spaces (Rodriguez et al., 2004). Rodriguez *et al.* (2004) suggest that the abnormal spongiotrophoblast arrangement may affect the development of the labyrinth either physically, by inhibiting branching of the maternal blood spaces by taking up physical space within the labyrinth, or molecularly, by secreting one or more factors which inhibit the normal behaviour of these cells (Rodriguez et al., 2004).

As a floxed allele of *Cited1* has already been prepared, this could be used to generate mice in which *Cited1* is inactivated only in the spongiotrophoblast layer of the placenta by crossing these mice to the Tpbpa-CIG mice. Although this approach may not clarify the question of whether the abnormal spongiotrophoblast arrangement results in labyrinth defects by a physical or molecular means, it would identify whether *Cited1* is required cell autonomously within the labyrinth layer, perhaps ruling out either possibility. If removing *Cited1* expression from the spongiotrophoblast cells restores normal labyrinth morphology, this would indicate that the defect observed in the labyrinth layer is a cell autonomous effect caused by the loss of *Cited1* in these cells. If the labyrinth defects are still apparent, this would support the supposition that the abnormal morphology of the spongiotrophoblast in the absence of *Cited1* affects the branching morphogenesis of the labyrinth.

Hypoxia inducible factor-1 (HIF-1) is a transcription factor composed of two subunits, the HIF-1a and aryl hydrocarbon receptor nuclear translocator (ARNT) basic helix loop helix proteins. The role of HIF-1 has been assessed by knocking out each of

the subunits separately. If either *HIF-1a* (Iyer et al., 1998; Ryan et al., 1998) or *ARNT* (Kozak et al., 1997) are knocked out, the embryos died *in utero* by E10.5 due primarily due to placental defects. *ARNT*-deficient mice demonstrate a loss of fetal vessel invasion with a related decrease in labyrinth formation and defects in spongiotrophoblast maintenance (Adelman et al., 2000; Kozak et al., 1997). A floxed allele of *ARNT* has been generated (Tomita et al., 2003) and could be useful in dissecting the role of *ARNT* in the labyrinth. Generating mice in which *ARNT* expression is only knocked out in the spongiotrophoblast could provide information about the nature of the defect in the labyrinth, as described for *Cited1* above.

There are many potential uses of the Tpbpa-CIG mice in studying placental development. These mice would not just be useful for further examining the role of genes which have already been knocked out throughout the placenta, but also for studying the role of genes that are known to be expressed in the spongiotrophoblast.

3.4 Conclusions

There are many different directions in which future work can continue. Cell lineage tracing of *Tpbpa*-expressing cells at earlier developmental stages will be approached as a priority, to examine both the route of endovascular invasion and how cell differentiation occurs within the EPC. Additional questions relating to cell lineage will require the generation of transgenic mice expressing Cre recombinase in other cell compartments, such as the extraembryonic ectoderm/chorion. Direct cell lineage tracing in the placenta will be useful in clarifying the relationship between the three main cell types of this tissue.

The Tpbpa-CIG mice can now be used with confidence to study cell specific genome alteration in the placenta, simply by choosing the transgenic line that is appropriate to the needs of the experiment. This will allow for the study of specific genetic elements in the spongiotrophoblast layer.

References

Abe, M., and Oshima, R. (1990). A single human keratin 18 gene is expressed in diverse epithelial cells of transgenic mice. *J. Cell Biol.* 111, 1197-1206.

Adamson, S. L., Lu, Y., Whiteley, K. J., Holmyard, D., Hemberger, M., Pfarrer, C., and Cross, J. C. (2002). Interactions between trophoblast cells and the maternal and fetal circulation in the mouse placenta. *Dev Biol* 250, 358-73.

Adelman, D. M., Gertsenstein, M., Nagy, A., Simon, M. C., and Maltepe, E. (2000). Placental cell fates are regulated in vivo by HIF-mediated hypoxia responses. *Genes Dev* 14, 3191-203.

Agah, R., Frenkel, P. A., French, B. A., Michael, L. H., Overbeek, P. A., and Schneider, M. D. (1997). Gene recombination in postmitotic cells. Targeted expression of Cre recombinase provokes cardiac-restricted, site-specific rearrangement in adult ventricular muscle in vivo. *J Clin Invest* 100, 169-79.

Akagi, K., Sandig, V., Vooijs, M., Van der Valk, M., Giovannini, M., Strauss, M., and Berns, A. (1997). Cre-mediated somatic site-specific recombination in mice. *Nucleic Acids Res* **25,** 1766-73

Amin, A., Roca, H., Luetke, K., and Sadowski, P. D. (1991). Synapsis, strand scission, and strand exchange induced by the FLP recombinase: analysis with half-FRT sites. *Mol Cell Biol* 11, 4497-508.

Beddington, R. S. (1982). An autoradiographic analysis of tissue potency in different regions of the embryonic ectoderm during gastrulation in the mouse. *J Embryol Exp Morphol* 69, 265-85.

Belteki, G., Gertsenstein, M., Ow, D. W., and Nagy, A. (2003). Site-specific cassette exchange and germline transmission with mouse ES cells expressing phiC31 integrase. *Nat Biotechnol* 21, 321-4.

Blaise, S., de Parseval, N., Benit, L., and Heidmann, T. (2003). Genomewide screening for fusogenic human endogenous retrovirus envelopes identifies syncytin 2, a gene conserved on primate evolution. *Proc Natl Acad Sci U S A* 100, 13013-8.

Bloxham, P.A., Pugh, D.M., and Sharma, S.C. (1977). Abolition of the pre-implantation surge of plasma oestrogens in mice with tamoxifen. *Irc Med Sci Reprod Obstet Gynecol* 5, 432.

Branda, C. S., and Dymecki, S. M. (2004). Talking about a revolution: The impact of site-specific recombinases on genetic analyses in mice. *Dev Cell* 6, 7-28.

Brocard, J., Warot, X., Wendling, O., Messaddeq, N., Vonesch, J. L., Chambon, P., and Metzger, D. (1997). Spatio-temporally controlled site-specific somatic mutagenesis in the mouse. *Proc Natl Acad Sci U S A* 94, 14559-63.

Buckley, R. H. (2004). Molecular defects in human severe combined immunodeficiency and approaches to immune reconstitution. *Annu Rev Immunol* 22, 625-55.

Calzonetti, T., Stevenson, L., and Rossant, J. (1995). A novel regulatory region is required for trophoblast-specific transcription in transgenic mice. *Dev Biol* 171, 615-26.

Carney, E. W., Prideaux, V., Lye, S. J., and Rossant, J. (1993). Progressive expression of trophoblast-specific genes during formation of mouse trophoblast giant cells in vitro. *Mol Reprod Dev* 34, 357-68.

Chang, C., Chen, P. T., Chang, G. D., Huang, C. J., and Chen, H. (2004). Functional Characterization of the Placental Fusogenic Membrane Protein Syncytin. *Biol Reprod*.

Cheon, Y. P., DeMayo, F. J., Bagchi, M. K., and Bagchi, I. C. (2004). Induction of cytotoxic T-lymphocyte antigen-2beta, a cysteine protease inhibitor in decidua: a potential regulator of embryo implantation. *J Biol Chem* 279, 10357-63.

Cinato, E., Mirotsou, M., and Sablitzky, F. (2001). Cre-mediated transgene activation in the developing and adult mouse brain. *Genesis* 31, 118-25.

Clark, A. F., Sharp, M. G. F., Morley, S. D., Fleming, S., Peters, J., and Mullins, J. J. (1997). Renin-1 Is Essential for Normal Renal Juxtaglomerular Cell Granulation and Macula Densa Morphology. *J. Biol. Chem.* 272, 18185-18190.

Colosi, P., Talamantes, F., and Linzer, D. I. (1987). Molecular cloning and expression of mouse placental lactogen I complementary deoxyribonucleic acid. *Mol Endocrinol* 1, 767-76.

Conley, A., and Hinshelwood, M. (2001). Mammalian aromatases. *Reproduction* 121, 685-95.

Copp, A. J. (1979). Interaction between inner cell mass and trophectoderm of the mouse blastocyst. II. The fate of the polar trophectoderm. *J Embryol Exp Morphol* 51, 109-20.

Copp, A. J. (1995). Death before birth: clues from gene knockouts and mutations. *Trends Genet* 11, 87-93.

Cross, J. C. (1998). Formation of the placenta and extraembryonic membranes. *Ann N Y Acad Sci* 857, 23-32.

Cross, J. C. (2000). Genetic insights into trophoblast differentiation and placental morphogenesis. *Semin Cell Dev Biol* 11, 105-13.

Cross, J. C., Anson-Cartwright, L., and Scott, I. C. (2002a). Transcription factors underlying the development and endocrine functions of the placenta. *Recent Prog Horm Res* 57, 221-34.

Cross, J. C., Baczyk, D., Dobric, N., Hemberger, M., Hughes, M., Simmons, D. G., Yamamoto, H., and Kingdom, J. C. (2003). Genes, development and evolution of the placenta. *Placenta* 24, 123-30.

Cross, J. C., Hemberger, M., Lu, Y., Nozaki, T., Whiteley, K., Masutani, M., and Adamson, S. L. (2002b). Trophoblast functions, angiogenesis and remodeling of the maternal vasculature in the placenta. *Mol Cell Endocrinol* 187, 207-12.

Cross, J. C., Werb, Z., and Fisher, S. J. (1994). Implantation and the placenta: key pieces of the development puzzle. *Science* 266, 1508-18.

Cruz, Y. P., and Pedersen, R. A. (1985). Cell fate in the polar trophectoderm of mouse blastocysts as studied by microinjection of cell lineage tracers. *Dev Biol* 112, 73-83.

Delaria, K., Fiorentino, L., Wallace, L., Tamburini, P., Brownell, E., and Muller, D. (1994). Inhibition of cathepsin L-like cysteine proteases by cytotoxic T-lymphocyte antigen-2 beta. *J Biol Chem* 269, 25172-7.

Deussing, J., Kouadio, M., Rehman, S., Werber, I., Schwinde, A., and Peters, C. (2002). Identification and characterization of a dense cluster of placenta-specific cysteine peptidase genes and related genes on mouse chromosome 13. *Genomics* 79, 225-40.

Doetschman, T., Gregg, R. G., Maeda, N., Hooper, M. L., Melton, D. W., Thompson, S., and Smithies, O. (1987). Targetted correction of a mutant HPRT gene in mouse embryonic stem cells. *Nature* 330, 576-8.

Downs, K. M. (1998). The murine allantois. *Curr Top Dev Biol* 39, 1-33.

Dragatsis, I., and Zeitlin, S. (2000). CaMKIIalpha-Cre transgene expression and recombination patterns in the mouse brain. *Genesis* 26, 133-5.

Escalante-Alcalde, D., Recillas-Targa, F., Hernandez-Garcia, D., Castro-Obregon, S., Terao, M., Garattini, E., and Covarrubias, L. (1996). Retinoic acid and methylation cis-regulatory elements control the mouse tissue non-specific alkaline phosphatase gene expression. *Mech Dev* 57, 21-32.

Fabian, J. R., Field, L. J., McGowan, R. A., Mullins, J. J., Sigmund, C. D., and Gross, K. W. (1989). Allele-specific expression of the murine Ren-1 genes. *J Biol Chem* 264, 17589-94.

Faria, T. N., Ogren, L., Talamantes, F., Linzer, D. I., and Soares, M. J. (1991). Localization of placental lactogen-I in trophoblast giant cells of the mouse placenta. *Biol Reprod* 44, 327-31.

Feil, R., Brocard, J., Mascrez, B., LeMeur, M., Metzger, D., and Chambon, P. (1996). Ligand-activated site-specific recombination in mice. *Proc Natl Acad Sci U S A* 93, 10887-90.

Fleming, T. P. (1987). A quantitative analysis of cell allocation to trophectoderm and inner cell mass in the mouse blastocyst. *Dev Biol* 119, 520-31.

Friedrich, G., and Soriano, P. (1991). Promoter traps in embryonic stem cells: a genetic screen to identify and mutate developmental genes in mice. *Genes Dev* 5, 1513-23.

Gossen, M., and Bujard, H. (1992). Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc Natl Acad Sci U S A* 89, 5547-51.

Graham, C. F., and Deussen, Z. A. (1978). Features of cell lineage in preimplantation mouse development. *J Embryol Exp Morphol* 48, 53-72.

Groth, A. C., Olivares, E. C., Thyagarajan, B., and Calos, M. P. (2000). A phage integrase directs efficient site-specific integration in human cells. *Proc Natl Acad Sci U S A* 97, 5995-6000.

Guillemot, F., Nagy, A., Auerbach, A., Rossant, J., and Joyner, A. L. (1994). Essential role of Mash-2 in extraembryonic development. *Nature* 371, 333-6.

Gustafsson, E., Brakebusch, C., Hietanen, K., and Fassler, R. (2001). Tie-1-directed expression of Cre recombinase in endothelial cells of embryoid bodies and transgenic mice. *J Cell Sci* 114, 671-6.

Hanssens, M., Vercruysse, L., Keirse, M. J., Pijnenborg, R., and Van Assche, F. A. (1995). Identification of 'renin'-containing cells in the choriodecidua. *Placenta* 16, 517-25.

Harada, N., Yoshimura, N., and Honda, S. (2003). Unique regulation of expression of human aromatase in the placenta. *J Steroid Biochem Mol Biol* 86, 327-34.

Hasan, M. T., Schonig, K., Berger, S., Graewe, W., and Bujard, H. (2001). Long-term, noninvasive imaging of regulated gene expression in living mice. *Genesis* 29, 116-22.

Hayashi, S., and McMahon, A. P. (2002). Efficient recombination in diverse tissues by a tamoxifen-inducible form of Cre: a tool for temporally regulated gene activation/inactivation in the mouse. *Dev Biol* 244, 305-18.

He, Y., Smith, S. K., Day, K. A., Clark, D. E., Licence, D. R., and Charnock-Jones, D. S. (1999). Alternative splicing of vascular endothelial growth factor (VEGF)-R1 (FLT-1) pre-mRNA is important for the regulation of VEGF activity. *Mol Endocrinol* 13, 537-45.

Hemberger, M., Nozaki, T., Masutani, M., and Cross, J. C. (2003). Differential expression of angiogenic and vasodilatory factors by invasive trophoblast giant cells depending on depth of invasion. *Dev Dyn* 227, 185-91.

Hesse, M., Franz, T., Tamai, Y., Taketo, M. M., and Magin, T. M. (2000). Targeted deletion of keratins 18 and 19 leads to trophoblast fragility and early embryonic lethality. *Embo J* 19, 5060-70.

Hoess, R., Abremski, K., and Sternberg, N. (1984). The nature of the interaction of the P1 recombinase Cre with the recombining site loxP. *Cold Spring Harb Symp Quant Biol* 49, 761-8.

Hoess, R. H., and Abremski, K. (1984). Interaction of the bacteriophage P1 recombinase Cre with the recombining site loxP. *Proc Natl Acad Sci U S A* 81, 1026-9.

Hoess, R. H., Ziese, M., and Sternberg, N. (1982). P1 site-specific recombination: nucleotide sequence of the recombining sites. *Proc Natl Acad Sci U S A* 79, 3398-402.

Hornbruch, A., Summerbell, D., and Wolpert, L. (1979). Somite formation in the early chick embryo following grafts of Hensen's node. *J Embryol Exp Morphol* 51, 51-62.

Hoshi, K., Amizuka, N., Oda, K., Ikehara, Y., and Ozawa, H. (1997). Immunolocalization of tissue non-specific alkaline phosphatase in mice. *Histochem Cell Biol* 107, 183-91.

Huang, Z. J., Yu, W., Lovett, C., and Tonegawa, S. (2002). Cre/loxP recombination-activated neuronal markers in mouse neocortex and hippocampus. *Genesis* **32,** 209-17.

Ihara, Y., Taii, S., and Mori, T. (1987). Expression of renin and angiotensinogen genes in the human placental tissues. *Endocrinol Jpn* 34, 887-96.

Indra, A. K., Warot, X., Brocard, J., Bornert, J. M., Xiao, J. H., Chambon, P., and Metzger, D. (1999). Temporally-controlled site-specific mutagenesis in the basal layer of the epidermis: comparison of the recombinase activity of the tamoxifen-

inducible Cre-ER(T) and Cre-ER(T2) recombinases. *Nucleic Acids Res* 27, 4324-7.

Iwatsuki, K., Shinozaki, M., Sun, W., Yagi, S., Tanaka, S., and Shiota, K. (2000). A novel secretory protein produced by rat spongiotrophoblast. *Biol Reprod* 62, 1352-9.

Iyer, N. V., Kotch, L. E., Agani, F., Leung, S. W., Laughner, E., Wenger, R. H., Gassmann, M., Gearhart, J. D., Lawler, A. M., Yu, A. Y., and Semenza, G. L. (1998). Cellular and developmental control of O2 homeostasis by hypoxia-inducible factor 1 alpha. *Genes Dev* 12, 149-62.

Jackson, L. L., Colosi, P., Talamantes, F., and Linzer, D. I. (1986). Molecular cloning of mouse placental lactogen cDNA. *Proc Natl Acad Sci U S A* 83, 8496-500.

Jones, C. A., Hurley, M. I., Black, T. A., Kane, C. M., Pan, L., Pruitt, S. C., and Gross, K. W. (2000). Expression of a renin/GFP transgene in mouse embryonic, extra-embryonic, and adult tissues. *Physiol Genomics* 4, 75-81.

Jones, C. A., Sigmund, C. D., McGowan, R. A., Kane-Haas, C. M., and Gross, K. W. (1990). Expression of murine renin genes during fetal development. *Mol Endocrinol* 4, 375-83.

Kamat, A., Graves, K. H., Smith, M. E., Richardson, J. A., and Mendelson, C. R. (1999). A 500-bp region, approximately 40 kb upstream of the human CYP19 (aromatase) gene, mediates placenta-specific expression in transgenic mice. *Proc Natl Acad Sci U S A* 96, 4575-80.

Kawamoto, S., Niwa, H., Tashiro, F., Sano, S., Kondoh, G., Takeda, J., Tabayashi, K., and Miyazaki, J. (2000). A novel reporter mouse strain that expresses enhanced green fluorescent protein upon Cre-mediated recombination. *FEBS Lett* **470,** 263-8.

Kellendonk, C., Tronche, F., Monaghan, A. P., Angrand, P. O., Stewart, F., and Schutz, G. (1996). Regulation of Cre recombinase activity by the synthetic steroid RU 486. *Nucleic Acids Res* 24, 1404-11.

Kisanuki, Y. Y., Hammer, R. E., Miyazaki, J., Williams, S. C., Richardson, J. A., and Yanagisawa, M. (2001). Tie2-Cre transgenic mice: a new model for endothelial cell-lineage analysis in vivo. *Dev Biol* 230, 230-42.

Kistner, A., Gossen, M., Zimmermann, F., Jerecic, J., Ullmer, C., Lubbert, H., and Bujard, H. (1996). Doxycycline-mediated quantitative and tissue-specific control of gene expression in transgenic mice. *Proc Natl Acad Sci U S A* 93, 10933-8.

Komminoth, P., Merk, F. B., Leav, I., Wolfe, H. J., and Roth, J. (1992). Comparison of 35S- and digoxigenin-labeled RNA and oligonucleotide probes for in situ hybridization. Expression of mRNA of the seminal vesicle secretion protein II and androgen receptor genes in the rat prostate. *Histochemistry* 98, 217-28.

Kozak, K. R., Abbott, B., and Hankinson, O. (1997). ARNT-deficient mice and placental differentiation. *Dev Biol* 191, 297-305.

Lan, Z. J., Xu, X., and Cooney, A. J. (2004). Differential oocyte-specific expression of Cre recombinase activity in GDF-9-iCre, Zp3cre, and Msx2Cre transgenic mice. *Biol Reprod* 71, 1469-74.

Lawson, K. A., Meneses, J. J., and Pedersen, R. A. (1991). Clonal analysis of epiblast fate during germ layer formation in the mouse embryo. *Development* 113, 891-911.

Lawson, K. A., and Pedersen, R. A. (1992). Clonal analysis of cell fate during gastrulation and early neurulation in the mouse. *Ciba Found Symp* 165, 3-21; discussion 21-6.

Le Du, M. H., and Millan, J. L. (2002). Structural evidence of functional divergence in human alkaline phosphatases. *J Biol Chem* 277, 49808-14.

Lemischka, I. R., Raulet, D. H., and Mulligan, R. C. (1986). Developmental potential and dynamic behavior of hematopoietic stem cells. *Cell* 45, 917-27.

Lescisin, K. R., Varmuza, S., and Rossant, J. (1988). Isolation and characterization of a novel trophoblast-specific cDNA in the mouse. *Genes Dev* 2, 1639-46.

Linzer, D. I., Lee, S. J., Ogren, L., Talamantes, F., and Nathans, D. (1985). Identification of proliferin mRNA and protein in mouse placenta. *Proc Natl Acad Sci U S A* 82, 4356-9.

Lo, C. W., Coulling, M., and Kirby, C. (1987). Tracking of mouse cell lineage using microinjected DNA sequences: analyses using genomic Southern blotting and tissue-section in situ hybridizations. *Differentiation* 35, 37-44.

Lobe, C. G., Koop, K. E., Kreppner, W., Lomeli, H., Gertsenstein, M., and Nagy, A. (1999). Z/AP, a double reporter for cre-mediated recombination. *Dev Biol* 208, 281-92.

Logie, C., and Stewart, A. F. (1995). Ligand-regulated site-specific recombination. *Proc Natl Acad Sci U S A* 92, 5940-4.

Lomeli, H., Ramos-Mejia, V., Gertsenstein, M., Lobe, C. G., and Nagy, A. (2000). Targeted insertion of Cre recombinase into the TNAP gene: excision in primordial germ cells. *Genesis* 26, 116-7.

Lu, X. J., Deb, S., and Soares, M. J. (1994). Spontaneous differentiation of trophoblast cells along the spongiotrophoblast cell pathway: expression of members of the placental prolactin gene family and modulation by retinoic acid. *Dev Biol* 163, 86-97.

Luo, J., Sladek, R., Bader, J. A., Matthyssen, A., Rossant, J., and Giguere, V. (1997). Placental abnormalities in mouse embryos lacking the orphan nuclear receptor ERR-beta. *Nature* 388, 778-82.

MacGregor, G. R., Zambrowicz, B. P., and Soriano, P. (1995). Tissue non-specific alkaline phosphatase is expressed in both embryonic and extraembryonic lineages during mouse embryogenesis but is not required for migration of primordial germ cells. *Development* 121, 1487-96.

Magin, T. M., Schroder, R., Leitgeb, S., Wanninger, F., Zatloukal, K., Grund, C., and Melton, D. W. (1998). Lessons from Keratin 18 Knockout Mice: Formation of Novel Keratin Filaments, Secondary Loss of Keratin 7 and Accumulation of Liver-specific Keratin 8-Positive Aggregates. *J. Cell Biol.* 140, 1441-1451.

Mao, X., Fujiwara, Y., Chapdelaine, A., Yang, H., and Orkin, S. H. (2001). Activation of EGFP expression by Cre-mediated excision in a new ROSA26 reporter mouse strain. *Blood* **97,** 324-6.

McLeod, M., Craft, S., and Broach, J. R. (1986). Identification of the crossover site during FLP-mediated recombination in the Saccharomyces cerevisiae plasmid 2 microns circle. *Mol Cell Biol* 6, 3357-67.

Metzger, D., Clifford, J., Chiba, H., and Chambon, P. (1995). Conditional site-specific recombination in mammalian cells using a ligand-dependent chimeric Cre recombinase. *Proc Natl Acad Sci U S A* 92, 6991-5.

Mi, S., Lee, X., Li, X., Veldman, G. M., Finnerty, H., Racie, L., LaVallie, E., Tang, X. Y., Edouard, P., Howes, S., Keith, J. C., Jr., and McCoy, J. M. (2000). Syncytin is a captive retroviral envelope protein involved in human placental morphogenesis. *Nature* 403, 785-9.

Mitsunaga, K., Araki, K., Mizusaki, H., Morohashi, K., Haruna, K., Nakagata, N., Giguere, V., Yamamura, K., and Abe, K. (2004). Loss of PGC-specific expression of the orphan nuclear receptor ERR-beta results in reduction of germ cell number in mouse embryos. *Mech Dev* 121, 237-46.

Nagy, A. (2000). Cre recombinase: the universal reagent for genome tailoring. *Genesis* 26, 99-109.

Nagy, A., Gocza, E., Diaz, E. M., Prideaux, V. R., Ivanyi, E., Markkula, M., and Rossant, J. (1990). Embryonic stem cells alone are able to support fetal development in the mouse. *Development* 110, 815-21.

Niwa, H., Yamamura, K., and Miyazaki, J. (1991). Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* 108, 193-9.

Novak, A., Guo, C., Yang, W., Nagy, A., and Lobe, C. G. (2000). Z/EG, a double reporter mouse line that expresses enhanced green fluorescent protein upon Cre-mediated excision. *Genesis* 28, 147-55.

Ogata, S., Hayashi, Y., Takami, N., and Ikehara, Y. (1988). Chemical characterization of the membrane-anchoring domain of human placental alkaline phosphatase. *J. Biol. Chem.* 263, 10489-10494.

O'Gorman, S., Dagenais, N. A., Qian, M., and Marchuk, Y. (1997). Protamine-Cre recombinase transgenes efficiently recombine target sequences in the male germ line of mice, but not in embryonic stem cells. *Proc Natl Acad Sci U S A* 94, 14602-7.

Ogren, L., and Talamantes, F. (1988). Prolactins of pregnancy and their cellular source. *Int Rev Cytol* 112, 1-65.

Oshima, R. G., Howe, W. E., Klier, F. G., Adamson, E. D., and Shevinsky, L. H. (1983). Intermediate filament protein synthesis in preimplantation murine embryos. *Dev Biol* 99, 447-55.

Parameswaran, M., and Tam, P. P. (1995). Regionalisation of cell fate and morphogenetic movement of the mesoderm during mouse gastrulation. *Dev Genet* 17, 16-28.

Petrovic, N., Black, T. A., Fabian, J. R., Kane, C., Jones, C. A., Loudon, J. A., Abonia, J. P., Sigmund, C. D., and Gross, K. W. (1996). Role of proximal promoter elements in regulation of renin gene transcription. *J Biol Chem* 271, 22499-505.

Poisner, A. M., Wood, G. W., Poisner, R., and Inagami, T. (1981). Localization of renin in trophoblasts in human chorion laeve at term pregnancy. *Endocrinology* 109, 1150-5.

Price, J. (1987). Retroviruses and the study of cell lineage. *Development* 101, 409-19.

Price, J., Turner, D., and Cepko, C. (1987). Lineage analysis in the vertebrate nervous system by retrovirus-mediated gene transfer. *Proc Natl Acad Sci U S A* 84, 156-60.

Rodriguez, T. A., Sparrow, D. B., Scott, A. N., Withington, S. L., Preis, J. I., Michalicek, J., Clements, M., Tsang, T. E., Shioda, T., Beddington, R. S. P., and Dunwoodie, S. L. (2004). Cited1 Is Required in Trophoblasts for Placental Development and for Embryo Growth and Survival. *Mol. Cell. Biol.* 24, 228-244.

Rossant, J., and Cross, J. C. (2001). Placental development: lessons from mouse mutants. *Nat Rev Genet* 2, 538-48.

Rossant, J., and Tamura-Lis, W. (1981). Effect of culture conditions on diploid to giant-cell transformation in postimplantation mouse trophoblast. *J Embryol Exp Morphol* 62, 217-27.

Ryan, H. E., Lo, J., and Johnson, R. S. (1998). HIF-1 alpha is required for solid tumor formation and embryonic vascularization. *Embo J* 17, 3005-15.

Sadowski, P. D. (1995). The Flp recombinase of the 2-microns plasmid of Saccharomyces cerevisiae. *Prog Nucleic Acid Res Mol Biol* 51, 53-91.

Sauer, B., and Henderson, N. (1988). Site-specific DNA recombination in mammalian cells by the Cre recombinase of bacteriophage P1. *Proc Natl Acad Sci U S A* 85, 5166-70.

Schwenk, F., Kuhn, R., Angrand, P. O., Rajewsky, K., and Stewart, A. F. (1998). Temporally and spatially regulated somatic mutagenesis in mice. *Nucleic Acids Res* 26, 1427-32.

Scott, I. C., Anson-Cartwright, L., Riley, P., Reda, D., and Cross, J. C. (2000). The HAND1 basic helix-loop-helix transcription factor regulates trophoblast differentiation via multiple mechanisms. *Mol Cell Biol* 20, 530-41.

Sharp, M. G. F., Fettes, D., Brooker, G., Clark, A. F., Peters, J., Fleming, S., and Mullins, J. J. (1996). Targeted Inactivation of the Ren-2 Gene in Mice. *Hypertension* 28, 1126-1131.

Shaw, K. J., Do, Y. S., Kjos, S., Anderson, P. W., Shinagawa, T., Dubeau, L., and Hsueh, W. A. (1989). Human decidua is a major source of renin. *J Clin Invest* 83, 2085-92.

Shi, D., Winston, J. H., Blackburn, M. R., Datta, S. K., Hanten, G., and Kellems, R. E. (1997). Diverse genetic regulatory motifs required for murine adenosine deaminase gene expression in the placenta. *J Biol Chem* 272, 2334-41.

Shida, M. M., Jackson-Grusby, L. L., Ross, S. R., and Linzer, D. I. (1992). Placental-specific expression from the mouse placental lactogen II gene promoter. *Proc Natl Acad Sci U S A* 89, 3864-8.

Shida, M. M., Ng, Y. K., Soares, M. J., and Linzer, D. I. (1993). Trophoblast-specific transcription from the mouse placental lactogen-I gene promoter. *Mol Endocrinol* 7, 181-8.

Sigmund, C. D., and Gross, K. W. (1991). Structure, expression, and regulation of the murine renin genes. *Hypertension* 18, 446-57.

Simmonds, H. A., Panayi, G. S., and Corrigall, V. (1978). A role for purine metabolism in the immune response: Adenosine-deaminase activity and deoxyadenosine catabolism. *Lancet* 1, 60-3.

Sladek, R., Beatty, B., Squire, J., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., and Giguere, V. (1997). Chromosomal mapping of the human and murine orphan receptors ERRalpha (ESRRA) and ERRbeta (ESRRB) and identification of a novel human ERRalpha-related pseudogene. *Genomics* 45, 320-6.

Smith, M. S. (1973). Changes in distribution of alkaline phosphatase during early implantation and development of the mouse. *Aust J Biol Sci* 26, 209-17.

Smith, R. L., Geller, A. I., Escudero, K. W., and Wilcox, C. L. (1995). Long-term expression in sensory neurons in tissue culture from herpes simplex virus type 1 (HSV-1) promoters in an HSV-1-derived vector. *J Virol* 69, 4593-9.

Soares, M. J. (2004). The prolactin and growth hormone families: pregnancy-specific hormones/cytokines at the maternal-fetal interface. *Reprod Biol Endocrinol* 2, 51.

Soares, M. J., Muller, H., Orwig, K. E., Peters, T. J., and Dai, G. (1998). The uteroplacental prolactin family and pregnancy. *Biol Reprod* 58, 273-84.

Sohal, D. S., Nghiem, M., Crackower, M. A., Witt, S. A., Kimball, T. R., Tymitz, K. M., Penninger, J. M., and Molkentin, J. D. (2001). Temporally regulated and tissue-specific gene manipulations in the adult and embryonic heart using a tamoxifen-inducible Cre protein. *Circ Res* 89, 20-5.

Sola, C., Tronik, D., Dreyfus, M., Babinet, C., and Rougeon, F. (1989). Renin-promoter SV40 large T-antigen transgenes induce tumors irrespective of normal cellular expression of renin genes. *Oncogene Res* 5, 149-53.

Soriano, P. (1999). Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat Genet* 21, 70-1.

Soriano, P., and Jaenisch, R. (1986). Retroviruses as probes for mammalian development: allocation of cells to the somatic and germ cell lineages. *Cell* 46, 19-29.

Srinivas, S., Watanabe, T., Lin, C. S., William, C. M., Tanabe, Y., Jessell, T. M., and Costantini, F. (2001). Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. *BMC Dev Biol* **1,** 4.

Stanley, E. G., Biben, C., Elefanty, A., Barnett, L., Koentgen, F., Robb, L., and Harvey, R. P. (2002). Efficient Cre-mediated deletion in cardiac progenitor cells conferred by a 3'UTR-ires-Cre allele of the homeobox gene Nkx2-5. *Int J Dev Biol* 46, 431-9.

St-Onge, L., Furth, P. A., and Gruss, P. (1996). Temporal control of the Cre recombinase in transgenic mice by a tetracycline responsive promoter. *Nucleic Acids Res* 24, 3875-7.

Tam, P. P., and Zhou, S. X. (1996). The allocation of epiblast cells to ectodermal and germ-line lineages is influenced by the position of the cells in the gastrulating mouse embryo. *Dev Biol* 178, 124-32.

Tanaka, M., Gertsenstein, M., Rossant, J., and Nagy, A. (1997). Mash2 acts cell autonomously in mouse spongiotrophoblast development. *Dev Biol* 190, 55-65.

Tanaka, M., Hadjantonakis, A. K., and Nagy, A. (2001). Aggregation chimeras. Combining ES cells, diploid and tetraploid embryos. *Methods Mol Biol* 158, 135-54.

Tanaka, S., Kunath, T., Hadjantonakis, A. K., Nagy, A., and Rossant, J. (1998). Promotion of trophoblast stem cell proliferation by FGF4. *Science* 282, 2072-5.

Thorey, I. S., Meneses, J. J., Neznanov, N., Kulesh, D. A., Pedersen, R. A., and Oshima, R. G. (1993). Embryonic expression of human keratin 18 and K18-beta-galactosidase fusion genes in transgenic mice. *Dev Biol* 160, 519-34.

Tickle, C., Goodman, M., and Wolpert, L. (1978). Cell contacts and sorting out in vivo: the behaviour of some embryonic tissues implanted into the developing chick wing. *J Embryol Exp Morphol* 48, 225-37.

Tomita, S., Jiang, H.-B., Ueno, T., Takagi, S., Tohi, K., Maekawa, S.-i., Miyatake, A., Furukawa, A., Gonzalez, F. J., Takeda, J., Ichikawa, Y., and Takahama, Y. (2003). T Cell-Specific Disruption of Arylhydrocarbon Receptor Nuclear

Translocator (Arnt) Gene Causes Resistance to 2,3,7,8-Tetrachlorodibenzo-p-dioxin-Induced Thymic Involution *J Immunol* 171, 4113-4120.

Tsien, J. Z., Chen, D. F., Gerber, D., Tom, C., Mercer, E. H., Anderson, D. J., Mayford, M., Kandel, E. R., and Tonegawa, S. (1996). Subregion- and cell type-restricted gene knockout in mouse brain. *Cell* **87,** 1317-26.

Uy, G. D., Downs, K. M., and Gardner, R. L. (2002). Inhibition of trophoblast stem cell potential in chorionic ectoderm coincides with occlusion of the ectoplacental cavity in the mouse. *Development* 129, 3913-24.

Wakamiya, M., Blackburn, M. R., Jurecic, R., McArthur, M. J., Geske, R. S., Cartwright, J., Jr., Mitani, K., Vaishnav, S., Belmont, J. W., and Kellems, R. E. (1995). Disruption of the adenosine deaminase gene causes hepatocellular impairment and perinatal lethality in mice. *Proc Natl Acad Sci U S A* 92, 3673-7.

Waymire, K. G., Mahuren, J. D., Jaje, J. M., Guilarte, T. R., Coburn, S. P., and MacGregor, G. R. (1995). Mice lacking tissue non-specific alkaline phosphatase die from seizures due to defective metabolism of vitamin B-6. *Nat Genet* 11, 45-51.

Wen, F., Cecena, G., Munoz-Ritchie, V., Fuchs, E., Chambon, P., and Oshima, R. G. (2003). Expression of conditional cre recombinase in epithelial tissues of transgenic mice. *Genesis* 35, 100-6.

Wessells, J., Wessner, D., Parsells, R., White, K., Finkenzeller, D., Zimmermann, W., and Dveksler, G. (2000). Pregnancy specific glycoprotein 18 induces IL-10 expression in murine macrophages. *Eur J Immunol* 30, 1830-40.

Wilson, I. B., Bolton, E., and Cuttler, R. H. (1972). Preimplantation differentiation in the mouse egg as revealed by microinjection of vital markers. *J Embryol Exp Morphol* 27, 467-9.

Wilson, V., and Beddington, R. S. (1996). Cell fate and morphogenetic movement in the late mouse primitive streak. *Mech Dev* 55, 79-89.

Winston, J. H., Hanten, G. R., Overbeek, P. A., and Kellems, R. E. (1992). 5' flanking sequences of the murine adenosine deaminase gene direct expression of a reporter gene to specific prenatal and postnatal tissues in transgenic mice. *J Biol Chem* 267, 13472-9.

Wood, S. A., Allen, N. D., Rossant, J., Auerbach, A., and Nagy, A. (1993). Non-injection methods for the production of embryonic stem cell-embryo chimaeras. *Nature* 365, 87-9.

Wooding, F. B. P. a. F., A.P.F. (1994). Placentation. *In* "Marshall's Physiology of Reproduction" (G. E. Lamming, Ed.), pp. 233-460. Chapman and Hall, New York.

Yan, J., Tanaka, S., Oda, M., Makino, T., Ohgane, J., and Shiota, K. (2001). Retinoic acid promotes differentiation of trophoblast stem cells to a giant cell fate. *Dev Biol* 235, 422-32.

Zambrowicz, B. P., Imamoto, A., Fiering, S., Herzenberg, L. A., Kerr, W. G., and Soriano, P. (1997). Disruption of overlapping transcripts in the ROSA beta geo 26 gene trap strain leads to widespread expression of beta-galactosidase in mouse embryos and hematopoietic cells. *Proc Natl Acad Sci U S A* 94, 3789-94.

Zybina, E. V., and Zybina, T. G. (1996). Polytene chromosomes in mammalian cells. *Int Rev Cytol* 165, 53-119.

Zinyk, D. L., Mercer, E. H., Harris, E., Anderson, D. J., and Joyner, A. L. (1998). Fate mapping of the mouse midbrain-hindbrain constriction using a site-specific recombination system. *Curr Biol* **8,** 665-8.