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A DNAJ-RELATED CO-CHAPERONE, MRJ, IS REQUIRED FOR PLACENTAL

DEVELOPMENT IN MICE

By

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# A DNAJ-RELATED CO-CHAPERONE, MRJ, IS REQUIRED FOR PLACENTAL DEVELOPMENT IN MICE

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## ABSTRACT

We have identified a new gene that plays a critical role in mouse placental development. This gene, called *m*ammalian *r*elative of Dna*J* (*Mrj*), was identified using gene trapping. *Mrj* is expressed in the trophoblast lineage at high levels and in specific embryonic and adult tissues at lower levels. The gene trap insertion created a hypomorphic allele of *Mrj*. In homozygous embryos, the allantois and the chorion fail to form an attachment which precludes normal placental development and results in death of the embryo at mid–gestation. *Mrj* encodes a protein that contains a J domain, so named due to its similarity with the Hsp70-interacting domain of *E. coli* DnaJ. Based on databases of expressed sequences, there are at least 40 J domain-containing proteins in mammals. The study of the *Mrj* represents the first genetic analysis of these proteins and has demonstrated a specific and unexpected role in placental development.

# ACKNOWLEDGEMENTS

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| | |
|---|---|
| BLAST | Basic local alignment search tool |
| cDNA | Complementary DNA |
| DNA | Deoxyribonucleic acid |
| E | Embryonic day |
| EPC | Ectoplacental cone |
| ES | Embryonic stem |
| EST | Expressed sequence tag |
| HSC | Heat shock cognate |
| HSE | Heat shock element |
| HSF | Heat shock factor |
| HSP | Heat shock protein |
| ICM | Inner cell mass |
| LTR | Long terminal repeats |
| mRNA | Messenger RNA |
| PCR | Polymerase chain reaction |
| Q-TOF | Quadrupole orthogonal acceleration - time of flight |
| RACE | Rapid amplification of cDNA ends |
| RNA | Ribonucleic acid |
| ROSA | Reverse orientation splice acceptor |
| snRNA | Small nuclear RNA |
| TS | Trophoblast stem |
| UTR | Untranslated region |

# CHAPTER ONE

# INTRODUCTION

## General Introduction

Development of the placenta is one of the most important processes of early prenatal life. Both human clinical studies and targeted mutagenesis experiments in mice support the hypothesis that much of fetal loss in eutherian mammals during pregnancy may be attributed to failure in proper development of this system (Copp, 1978; Cross et al., 1994; Wilcox et al., 1988). The placenta allows the conceptus to implant into the uterus of its mother and is the site of exchange of gases nutrients and waste between the mother and fetus during pregnancy.

Genes that are critical for proper development of the placenta are beginning to be identified. Mutational analysis in the mouse has enabled many of the cell proliferation and differentiation events to be attributed to the function of specific regulatory factors. Most of these factors were identified as candidate genes with placental expression. Due to the fact that mammals cannot be readily observed as they develop, genetic screens for placental defects are both daunting and impractical. Screens that identify genes based on expression patterns are much more easily undertaken and one very useful screening tool is gene trapping in murine embryonic stem cells. Our collaborator, Dr. Gary Lyons (University of Wisconsin) performed a large scale gene trapping screen using the ROSAβgeo gene trapping vector. From the 6AD1 cell line produced in this screen, a gene was cloned that was found to be expressed in trophoblast cells of the placenta using in situ hybridization techniques. Sequencing the gene revealed that it was novel but contained a domain that identified it with a large family of "co-chaperone" proteins. The domain shared by this family is known as a J domain because it was discovered in *E. coli* DnaJ. Members of the Hsp70 family require J domain-containing proteins for their chaperone activity. The gene was named *m*ammalian *r*elative of Dna*J* (*Mrj*).

Our work has shown that *Mrj* is required for the development of the mouse placenta. In this work, the studies of placental development and chaperone function are

brought together. A review of these subjects as well as a description of the gene trapping system that led to the discovery of *Mrj* will be presented in this introduction.

## Part 1: Placental Development

The placenta is the first organ to develop in mammals and is required throughout gestation to sustain the fetus. It is primarily derived from two distinct cell lineages: mesoderm and trophoblast. The mesodermal cells differentiate into the blood vessels and also the stromal cells which support the feto-placental circulation. The trophoblast cells lie at the periphery of the conceptus and thus interface directly with the mother. They function to transport nutrients and gases to the fetus, but also have special endocrine and immunological properties that affect maternal physiological function (Cross et al., 1994).

### Early Development of the Trophoblast Lineage

The trophoblast cell lineage is the first to be specified during mammalian embryonic development. It forms at the morula-to-blastocyst transition. Cells on the outside of the morula, which are fated to become trophoblast, form a simple epithelium (trophectoderm) which becomes the outer monolayer of the blastocyst (Figure 1). At this early stage, the developmental potential of these cells is restricted to trophoblast derivatives (Gardner and Johnson, 1972). The cells remaining on the inside of the blastocyst, the inner cell mass (ICM), are pluripotent stem cells that will give rise to the embryo proper as well as to placental mesoderm and other extraembryonic membranes. While embryonic development from the blastocyst stage is similar among mammals, extraembryonic development differs substantially. Detailed description will therefore be restricted to the mouse.
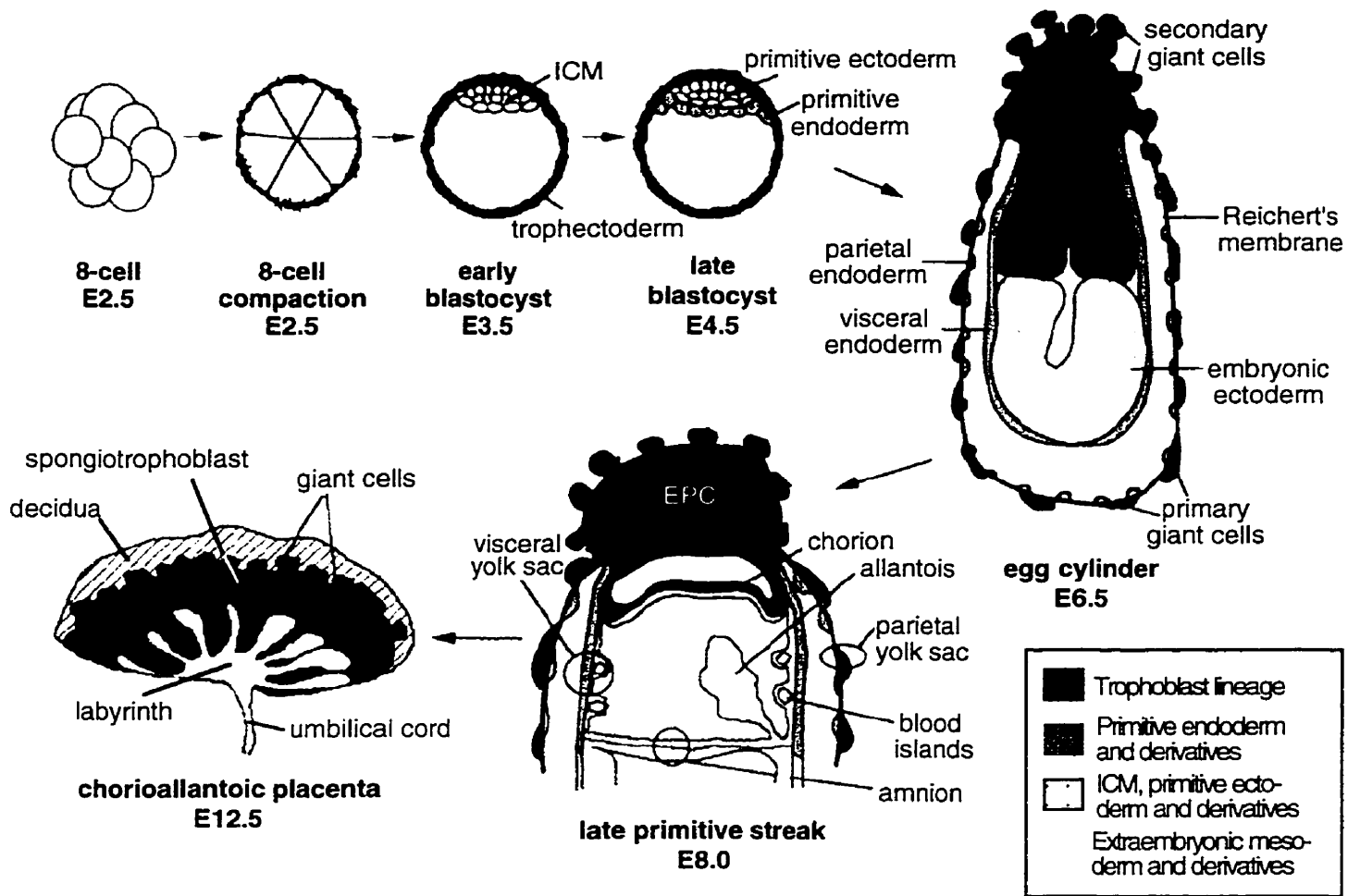
**Figure 1. Development of extraembryonic cell lineages.** E, gestational age (eg. E2.5 = embryonic day 2.5). Adapted from Rossant (1995).

In the mouse, the developmental fate of trophectoderm is controlled by its position within the blastocyst. The mural trophectoderm, that which is not in contact with the ICM, stops dividing at implantation at embryonic day 4.5 (E4.5). Cell growth and DNA synthesis in the absence of cell division occurs in these cells and by E6.5, they appear "giant"; large cells with polyploid nuclei. The polar trophectoderm, which is in contact with the ICM, continues to proliferate creating a mass of trophoblast called the ectoplacental cone (EPC). Cells on the periphery of the EPC differentiate into secondary trophoblast giant cells. Contact with the ICM and later the embryonic ectoderm is essential to the proliferation of the EPC because it will differentiate into giant cells in vitro if isolated from embryonic tissue (Gardner and Johnson, 1972; Rossant and Ofer, 1977). Presumably, a signal emanating from the embryonic tissue is acting as a mitogen. Trophoblast cells of the EPC adjacent to the embryonic ectoderm form the future chorion. The chorion contributes to the labyrinthine layer of the mature placenta. The EPC becomes the spongiotrophoblast layer. Both the EPC and the spongiotrophoblast layer contribute new (secondary) giant cells to the maternal-fetal interface (Figure 1).

Recent molecular genetic studies in mice have suggested that the development of the different trophoblast subtypes is regulated by different transcription factors (Figure 2). The earliest acting transcription factor gene is *Cdx2*. Expression of this homeobox gene is restricted to the trophectoderm at E3.5 (Beck et al., 1995). *Cdx2* has several other functions during embryogenesis based on the fact that heterozygous mice have phenotypic abnormalities (Chawengsaksophak et al., 1997). The precise function of *Cdx2* in the trophectoderm is unknown. *Cdx2* homozygous mutant embryos were reported to form blastocysts but fail shortly thereafter. At face value then, *Cdx2* may not be required for establishment of the trophoblast lineage. However, factors which initially specify trophoblast function may be the products of maternal transcripts present in the oocyte that mask the requirement of a zygotic factor until later in development.
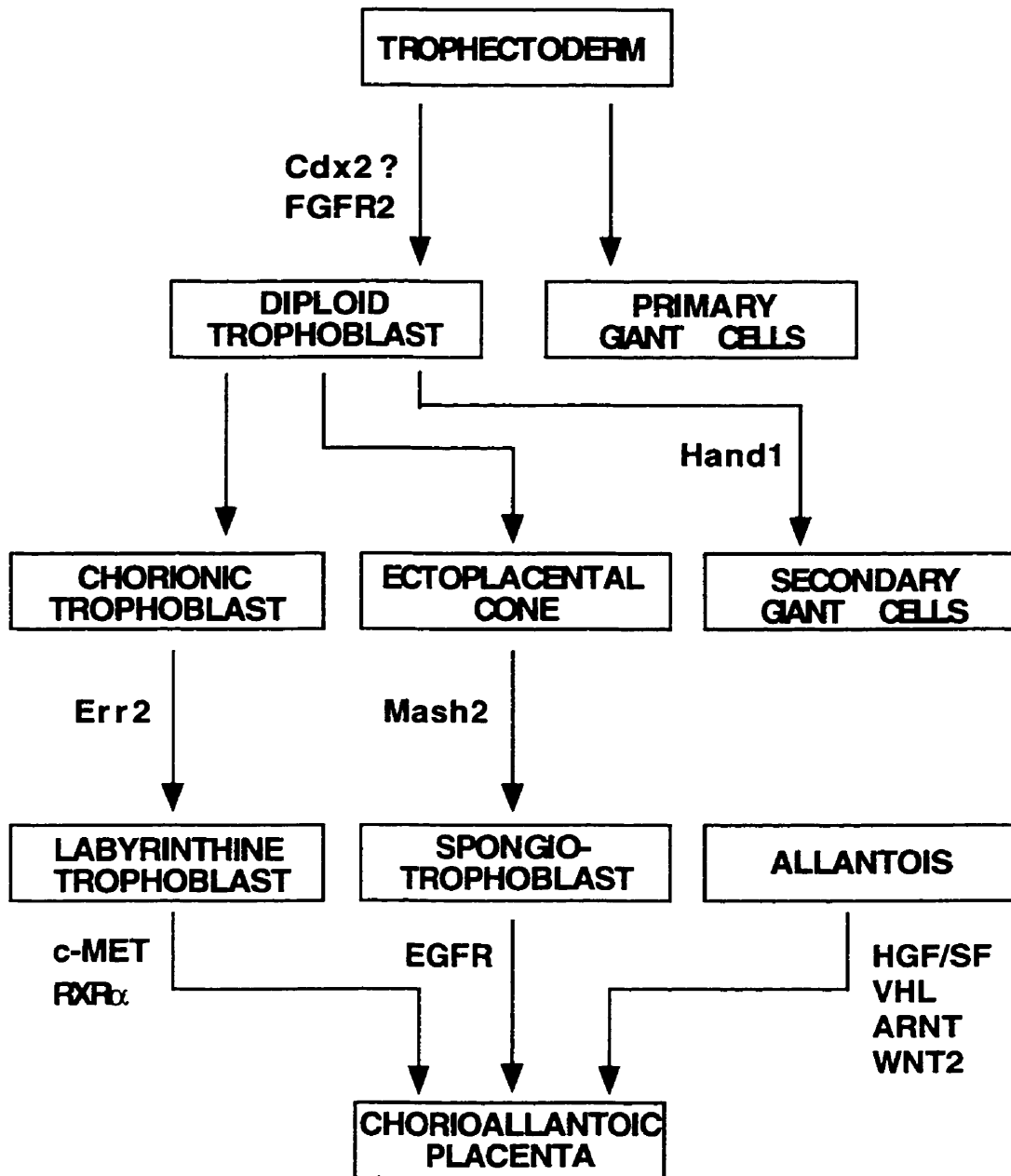
**Figure 2. Cell lineage development during formation of the placenta.** Genes with critical roles in developmental steps are shown. "Allantois" box is shaded to distinguish it from the trophoblast lineage. Adapted from Rossant *et al.* (1998).

*Err2* encodes an orphan nuclear receptor with greatest similarity to the estrogen receptor (Pettersson et al., 1996). *Err2*-deficient embryos die due to early trophoblast failure (Luo et al., 1997). Beyond E6.5, mutants lack chorionic structures and they die with small placentas by mid gestation. *Err2* is normally expressed in the chorionic trophoblast cells and therefore, it is likely that *Err2* is required to sustain their proliferation.

*Mash2* encodes a basic helix-loop-helix transcription factor that plays an essential role in the development of the spongiotrophoblast layer. *Mash2* deficient embryos appear normal until around E8. By E10.5, however, the spongiotrophoblast is absent (Guillemot et al., 1994). The precise defect is unknown, though it is thought that diploid trophoblast cells of the ectoplacental cone differentiate prematurely into trophoblast giant cells. This interpretation suggests that *Mash2* is required to maintain trophoblast cells in a proliferating, undifferentiated state. *Mash2* function is restricted to the trophoblast lineage. The requirement for *Mash2* can be circumvented by aggregation with wild type tetraploid embryos which contribute cells to the trophoblast lineage. *Mash2* null mice produced by tetraploid aggregation have no apparent defects (Guillemot et al., 1994). They have been mated to test for a role for maternal *Mash2* in embryogenesis. Mutant embryos produced from *Mash2* null females have the same phenotype as *Mash2* null embryos from heterozygous mothers (Rossant et al., 1998). This result indicates that although maternal *Mash2* transcripts are present in the oocyte and two-cell stage embryos, its function is not required. *Mash2* is paternally imprinted; the paternal allele is transcriptionally "silenced" in the embryo starting at around implantation (Guillemot et al., 1995; Rossant et al., 1998). Therefore, the homozygous mutant phenotype is observed in heterozygous embryos where the mutant allele is from the mother.

Defects in the spongiotrophoblast layer can also be seen with a deficiency of the epidermal growth factor receptor (*Egfr*) in an inbred mouse background. Unlike the *Mash2* mutants, the spongiotrophoblast layer is present in *Egfr* mutants, but is greatly

reduced in size (Sibilia and Wagner, 1995). This could explain why *Egfr* mutants survive until E12.5 which is longer than *Mash2* mutants. The placental defect of *Egfr* mutants appears to be a failure of spongiotrophoblast proliferation. In addition, the placenta was found to separate easily from the decidua suggesting a trophoblast cell adhesion defect (Sibilia and Wagner, 1995). In support of this hypothesis, *Egfr* has been reported to associate with cell adhesion molecules in vitro (Hoschuetzky et al., 1994).

*Hand1* (formerly known as *Hxt/eHAND*) encodes another basic-helix-loop-helix transcription factor involved in trophoblast giant cell differentiation. *Hand1* null embryos die starting at around E8 with a reduction in the number of trophoblast giant cells (Riley et al., 1998). Primary giant cells are present in *Hand1* null embryos. However, their nuclear size and expression of *Pl1*, a giant cell marker, are greatly reduced (P. Riley and J. Cross, unpublished data). It is possible, therefore, that initial differentiation of these cells may be rescued by maternal *Hand1* transcripts in the preimplantation embryo (Cross et al., 1995). Unfortunately, this question is difficult to address because tetraploid aggregation does not rescue mutant embryos due to the requirement for *Hand1* in cardiac morphogenesis (Riley et al., 1998). In the trophoblast lineage, *Hand1* seems to promote differentiation to the giant cell fate, an activity that opposes the activity of *Mash2*. In *Hand1* null embryos, the domain of *Mash2* expression is expanded, suggesting that the downregulation of *Mash2* is either necessary for or a consequence of trophoblast differentiation (Riley et al., 1998).

**Development of the Allantois**

During gastrulation, which begins around E6.5, cells of the embryonic ectoderm pass through the primitive streak and become mesoderm. Cells at the most posterior end of the primitive streak become extraembryonic mesoderm which gives rise to the amnion, which divides the pro-amniotic cavity into the amniotic and exocoelomic cavities, and the

visceral yolk sac, where blood islands form (Figure 1). A bud of mesodermal cells moving into the exocoelomic cavity towards the end of gastrulation forms the allantois. It takes on a distinct bulbous shape which occupies approximately one-third of the exocoelomic space at E8. By about E8.5, the allantois attaches to the chorion.

In general, genes that have an effect on mesoderm development affect the outgrowth of extraembryonic mesoderm as well. For example, mutations of the *DNmT* and *Lim1* genes have broad effects on mesoderm proliferation (Li et al., 1992; Shawlot and Behringer, 1995) and *brachyury* mutants which show abnormal mesoderm patterning (Gluecksohn-Schoenheimer, 1944). Mutations in these genes preclude chorioallantoic attachment most likely because of insufficient outgrowth of the mesoderm. In the case of *brachyury*, cell adhesion may be altered as well (Downs, 1998). Some genes have more specific roles in mesoderm development. An example is *FGFR1* which encodes a receptor for some members of the fibroblast growth factor family of the signaling molecules. While embryos which lack *FGFR1* are defective in embryonic mesoderm patterning, the extraembryonic mesoderm of the allantois, amnion and yolk sac is normal (Deng et al., 1994; Yamaguchi et al., 1994).

**Formation of the Placental Labyrinth**

The labyrinthine layer of the placenta is formed by the interaction of the allantois with the chorion and their subsequent concerted morphogenesis. The initiating event is chorioallantoic attachment at E8.5 for which specific cell adhesion molecules are required. Mutation in either the *α4 integrin* or *VCAM1* genes results in failure of chorioallantoic attachment (Gurtner et al., 1995; Kwee et al., 1995; Yang et al., 1995). VCAM1 is expressed in the distal two-thirds of the allantois about six hours before it contacts the chorion (Kwee et al., 1995). α4 integrin, which is a receptor for VCAM1, is expressed on the surface of the chorion from a much earlier time point (Kwee et al.,

1995; Yang et al., 1995). While these mutant phenotypes indicate that the VCAM1/α4 interaction is important, neither phenotype is completely penetrant. In some mutants, chorioallantoic fusion can occur indicating that other attachment molecules or mechanisms exist (Gurtner et al., 1995; Kwee et al., 1995).

FGFR2 is a cell surface receptor for several members of the fibroblast growth factor family which functions to transduce their mitogenic signals. Embryos lacking FGFR2 die shortly after implantation (Arman et al., 1998). However, mice that are homozygous for a partially functional allele survive until mid-gestation and develop placental defects (Xu et al., 1998). One-third of the homozygous mutant embryos fail to undergo chorioallantoic attachment. The other two-thirds have allantoises which attach and even vascularize but there is no labyrinth development. The precise role of FGFR2 in chorioallantoic attachment is not known. However, the later phenotype was attributed to the lack of chorionic cell proliferation (Xu et al., 1998). FGFR2, which is expressed in the trophoblast lineage, appears to promote the rapid proliferation of the chorionic trophoblast cells during labyrinth development.

The labyrinth contains a large network of fetal blood vessels and maternal blood sinuses separated by a three layered trophoblast structure (Wooding and Flint, 1994). It develops following chorioallantoic fusion by complex outgrowth and folding of the chorioallantoic plate thus creating a large surface area for nutrient transport and gas exchange. This epithelial-mesenchyme interaction and co-ordinated morphogenesis is similar to the formation of crypts and villi in the intestine and to branching morphogenesis in the lungs, kidney, mammary gland and pancreas. Hepatocyte growth factor/scatter factor (HGF/SF) and its receptor, c-MET, have been implicated in all of these processes (Kolatsi-Joannou et al., 1997). Embryos which lack either HGF/SF or c-MET die at around E13 due to a placental defect (Bladt et al., 1995; Schmidt et al., 1995; Uehara et al., 1995). HGF/SF is expressed by the allantois and is presumed to affect the chorion, which expresses c-MET. While chorioallantoic fusion occurs in mutant

embryos, labyrinthine development is reduced. It is likely that insufficiency of the exchange surface results in the death of the embryo as the demands made on the placenta increase with gestational age. However, the phenotype is complicated by the putative role of the HGF/SF in blood vessel branching (angiogenesis) (Rosen et al., 1993). Mutant placentas also show reduced vascularization of the labyrinth which could be the result of failed angiogenesis or a secondary result of the failed labyrinthine morphogenesis.

For the remainder of gestation, the primary function of the labyrinth is the import of nutrients and the export of waste from the fetal circulation. Much is known about the physiology of these processes but very little is known about the genes that function in the placenta during this period of gestation. Mutations of the *Wnt2* (Monkley et al., 1996) and *RXRα* (Sapin et al., 1997) genes result in severe placental disruption beginning at E12.5. Both phenotypes include edema, fibrinoid deposits in the labyrinth and disruption of fetal circulation leading to blood pooling. It is not clear whether these genes affect the morphogenesis or the function of the labyrinth, or both. In the case of *RXRα*, a lack of lipid droplets in the labyrinthine trophoblast cells was suggestive of a lipid transport defect (Sapin et al., 1997).

## Vascularization of the Placenta

The origin of the placental blood vessels remains a matter of some debate. Previously, the absence of endothelial cells in the allantois was taken to indicate that its vascularization occurred by angiogenesis or the branching of blood vessels from the embryo (Risau and Flamme, 1995). However, it has been speculated that blood island formation (hemangiogenesis) can occur in the allantois in the absence of primitive endoderm (Caprioli et al., 1998; Downs, 1998). Vascularization would then occur by the coalescence of blood islands (vasculogenesis). Beyond the origin of the vessels, the molecular mechanisms underlying their formation appear in some respects to be different

than that of the yolk sac or embryo. There are no mouse mutants reported to date that show normal placental vascularization in the absence of any yolk sac vascularization. In contrast, *ARNT* (Kozak et al., 1997) and *VHL* (Gnarra et al., 1997) mutants show limited placental vascularization with no apparent effects on the vascularization of the yolk sac or embryo even though these genes are also expressed in these sites.

## Placental Development in Other Species

The gross anatomical structure of the placenta differs substantially between species, perhaps more than any other organ (Wooding and Flint, 1994). The large surface area of the maternal-fetal interface in the placenta is common to all species. The two features that differ among species and are used for categorization are shape and number of cell layers between the maternal and fetal circulations. In the pig and the horse the shape of the placenta is termed diffuse because it covers the entire surface of the conceptus. In the pig, chorionic villi interdigitate with endometrial villi of the uterus. In the horse, villous projections extend from the chorionic girdle which aid in both the attachment and invasion of the embryo into the uterine epithelium. Pig and horse placentas are epitheliochorial because exchange of nutrients takes place across the uterine epithelium and chorionic trophoblast in addition to the vascular endothelia of both mother and fetus.

Sheep, cows and deer have evolved a placenta which attaches to the uterus by forming cotyledons around the uterine caruncles. Although the layer of trophoblast cells undergoes expansion and morphogenesis in the cotyledon, the overall exchange surface attained by the cotyledon-caruncle interaction is probably the lowest per fetal weight in these species. However, exchange is optimized by the cellular fusion of the fetal binucleate trophoblast cells with the maternal epithelial cells so that exchange may occur over a single syncytium instead of two distinct membranes.

In primates and probably most eutherian species, mesoderm underlies trophoblast from an early time point in gestation. The development of the murine placenta differs in this respect. The trophoblast lineage develops in the absence of contact with mesoderm for the first third of gestation until outgrowth of mesoderm from the embryo culminates in the formation of the labyrinth. Despite this difference in early development, both primates and rodents have discoid hemochorial placentas which consist of a syncytial trophoblast layer exposed to maternal blood on one side and fetal mesenchyme containing blood vessels on the other side. The chorionic villi of the human placenta float in maternal blood in the intervillous space.

The molecular mechanisms for the expansion of the placental surface area may be conserved across species. HGF/SF and its receptor c-MET, as described above, are involved in labyrinth development in mice. They are also expressed in the developing placentas of other species. In humans, HGF/SF is expressed in the placental mesenchyme and c-MET is expressed in the cytotrophoblast layer (Kolatsi-Joannou et al., 1997). In the horse, c-MET is expressed in the trophoblast layer overlying the villous projections which grow out from the chorionic girdle and HGF/SF is expressed in the underlying mesenchyme (Stewart, 1996). These expression patterns suggest that HGF/SF and c-MET may be involved in the morphogenesis of the vascular exchange structure of the placenta in all species.

**Part 2: Gene Trapping**

Gene trapping is a method for identifying genes. It involves the random insertion of a DNA sequence tag into the genome which can be used for selection and subsequent cloning. A very useful variation of this technique uses a gene tag encoding a reporter which can therefore be used to monitor endogenous gene expression. By insertion into the genome the construct may disrupt the function of the targeted gene and thus produce a phenotype. As such, gene trapping can be used as a screen in which both reporter expression and/or phenotypic consequences can be monitored. In mice, this technique is often practiced using murine embryonic stem (ES) cells due to the cost of screening individual mice directly for a phenotype. Some characterization of the trapped gene is often carried out in vitro before the decision is made to introduce the mutation into the germline.

**Use of the ROSAβgeo Gene Trap Vector in Embryonic Stem Cells**

The ROSAβgeo gene trapping system is one of the most widely used and has made a significant contribution to the identification of new genes involved in mammalian development (Evans, 1998). The ROSAβgeo cassette lacks a promoter and, therefore, will be expressed only if it inserts into a transcriptionally active gene (Figure 3). The 5' end of ROSAβgeo cassette contains a splice acceptor so that it will likely be expressed as a fusion transcript with the endogenous transcript. *βgeo* encodes a fusion protein representing β-galactosidase and neomycin resistance enzymes. After selection with G418 (a neomycin derivative), surviving ES cell clones should express β-galactosidase activity which can be detected by histochemical staining with X-gal. The placement of the polyadenylation signal at the 3' end of the βgeo gene is for the purpose of truncating

the transcript. Provided that no readthrough occurs, production of the endogenous transcript is terminated by ROSAβgeo at the point where it inserts.

There are two general methods of introducing the gene trapping vector into the embryonic stem cell line. The first involves electroporation of the cells with linearized plasmid DNA and the second involves infection of the cells with a retrovirus containing the vector. The retroviral approach has two main advantages. First, the retrovirus infects at a rate of one particle per cell which eliminates multiple integrations at different sites or as concatemers within a single locus (Friedrich and Soriano, 1993). Second, the provirus inserts cleanly and, in most cases, completely into the genome without causing deletions or rearrangements (Friedrich and Soriano, 1993). This facilitates the cloning of cDNA and genomic DNA from the inserted locus.

One main disadvantage of the retroviral approach is that the long terminal repeat (LTR) DNA sequence of most viruses has enhancer activity (Friedrich and Soriano, 1991). These sequences flank the gene trap vector and facilitate its integration into the host genome. Design of an improved retroviral vector by Friedrich and Soriano involved deletion of cis-acting elements from the LTRs without compromise of integration potential (Friedrich and Soriano, 1991). In addition, the LTRs were placed in reverse orientation with respect to the βgeo cassette.

The power of using ES cells for gene trapping is that clones can be easily introduced into mice using aggregation chimera technology (Figure 3) (Gossler and Zachgo, 1993). Expression of the trapped gene can be studied by staining whole embryos or histological sections for β-galactosidase activity. In addition, the effect of the insertion on endogenous gene function may be studied by looking for a phenotype in mice carrying the ROSAβgeo allele. Roughly 30% of ROSAβgeo cell lines produce mice with heterozygous or homozygous mutant phenotypes (Friedrich and Soriano, 1991)

Initial characterization of a ROSAβgeo ES cell line may be carried out by testing for patterns of reporter gene expression during in vitro differentiation (Figure 3). The
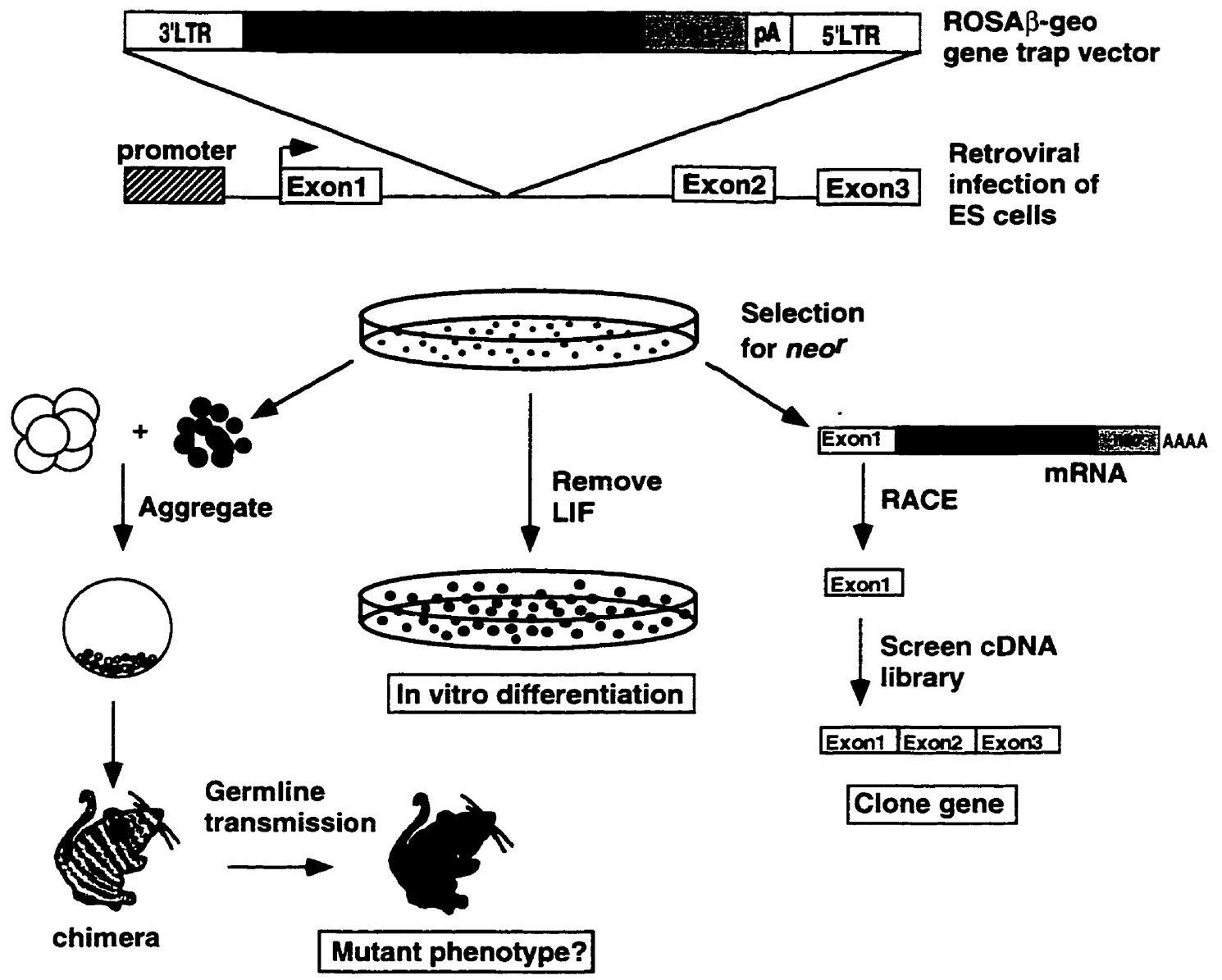
**Figure 3. Schematic representation of gene trapping strategy.**

removal of leukemia inhibitory factor (LIF) from the standard ES cell culture media and plating the dispersed ES cells on bacteriological dishes for a few days induces the formation of embryoid bodies which continue to develop, laying down embryonic germ layers. These bodies can be left floating or replated into tissue culture plates to which they will attach. The culture media can be altered to bias, to a limited extent, towards the induction of different cell types including hematopoetic, muscle, neurons and glia (Baker et al., 1997). In vitro differentiation and β-galactosidase staining permits the preselection of cell clones for gene expression patterns of interest.

Identification of the trapped gene is commonly accomplished using rapid amplification of cDNA ends (RACE), a form of RT-PCR (Figure 3). RNA from the ROSAβgeo ES cell line is isolated and primed for reverse transcription using an oligonucleotide that is complementary to the 5' end of *βgeo*. Reverse transcriptase will then polymerize cDNA complementary to the exons upstream of the gene trap insertion. An adapter sequence is ligated to the end of the resulting cDNA. The sequence is then amplified using the *βgeo* primer and a primer directed to the adapter sequence. The RACE product is then sequenced and if the sequence is unique, it can be used as a probe to screen a cDNA library in order to obtain the full length cDNA.

RACE products from ROSAβgeo ES cell lines tend to constitute the 5' untranslated regions of the endogenous genes (Friedrich and Soriano, 1991). This occurs because of a bias in ROSAβgeo design towards the production of cell lines with insertions upstream from the ATG. If insertion occurs downstream of the ATG, there is only a one in three probability that the βgeo will be spliced in frame with the endogenous gene's open reading frame enabling the cell line to survive selection; out-of-frame insertions will be therefore selected against.

**Part 3: Molecular Chaperones**

Molecular chaperones are proteins that assist other proteins in achieving or maintaining their active conformations. They bind to exposed hydrophobic residues in their substrates that would be otherwise buried if they were correctly folded (Hartl, 1996). Unfolded states of proteins occur during their translation, translocation, and degradation, or may be the consequence of cellular stress such as following heat shock. Chaperones have intrinsic ATPase activity that is essential to drive the conformational changes required for interaction with their substrates. "Co-chaperones" are proteins that assist or stimulate the activity of chaperones but do not selectively bind unfolded proteins.

There are two general classes of chaperones found in most organisms, both prokaryotic and eukaryotic (Hsp70 and Cpn60 type) (Table 1). Highly conserved members of these two families have been found in all organisms examined to date except *Methanococcus jannaschii* (Bult et al., 1996) which is a representative member of the archeabacteria phylum. The most well-characterized chaperones are DnaK (an Hsp70) and GroEL (a Cpn60) which were discovered in *E. coli* as host factors involved in phage replication. Despite the broad range of chaperone activity in *E. coli*, the bacterium remains viable without chaperones, however, it is phage replication incompetent. DnaK is required for recruitment of host DNA polymerase to the phage origin of replication (Osipiuk et al., 1993; Wall and Georgopoulos, 1993). GroEL is necessary for folding and assembly of phage coat proteins (Tilly et al., 1981).

**Hsp70 Chaperone Activity**

It is now firmly established the dominant force in protein folding is the mutual attraction of hydrophobic residues and their repulsion from the surrounding aqueous

**Table 1. Hsp70 and Cpn60 chaperones and DnaJ co-chaperones in *E.coli*, *S. cerevisiae* and mammals**

| Class | Protein | Organism | Organelle/ Cell Type | Phenotype | Reference |
|-------|---------|----------|---------------------|-----------|-----------|
| Hsp70 | DnaK | *E. coli* | | thermosensitive, phage replication - | (Georgopoulos et al., 1979) |
| | Ssa | *S. cerevisiae* | cytosol | thermosensitive | (Craig and Jacobsen, 1985) |
| | Ssb | *S. cerevisiae* | cytosol | slow growing | (Nelson et al., 1992) |
| | BiP/Kar2/Lhs1 | *S. cerevisiae* | ER | defective secretion | (Munro and Pelham, 1986) |
| | mtHSP70/Ssc | *S. cerevisiae* | mitochondria | not viable | (Scherer et al., 1990) |
| | Hsp90 | *S. cerevisiae* | cytosol | pleotropic | (Finkelstein and Strausberg, 1983) |
| | HSP70-1 | mammals | cytosol | | (Milner and Campbell, 1990) |
| | HSP70-2 | mammals | male germ cells | male infertility (mouse) | (Dix et al., 1996) |
| | HSP70-3 | mammals | cytosol | | (Grosz et al., 1992) |
| | HSP70t | mammals | postmeiotic male germ cells | | (Allen et al., 1988) |
| | HSC70 | mammals | cytosol | | (Ignolia et al., 1982) |
| Cpn60 | GroEL | *E. coli* | | thermosensitive phage replication - | (Tilly et al., 1981) |
| | TCP-1 | *S. cerevisiae* | cytosol | | (Frydman et al., 1994) |
| DnaJ | DnaJ | *E. coli* | | thermosensitive phage replication - | (Sunshine et al., 1977) |
| | Sec63 | *S. cerevisiae* | ER | defective secretion | (Sadler et al., 1989) |
| | Scj1 | *S. cerevisiae* | ER | defective secretion | (Schlenstedt et al., 1995) |
| | Sis1 | *S. cerevisiae* | cytosol | not viable | (Luke et al., 1991) |
| | Mdj | *S. cerevisiae* | mitochondria | | (Westermann et al., 1996) |
| | Ydj1 | *S. cerevisiae* | cytosol | slow growing | (Caplan and Douglas, 1991) |
| | HSP40 | mammals | cytosol | | (Raabe and Manley, 1991) |
| | HDJ2 | mammals | cytosol | | (Chellaiah et al., 1993) |
| | HSJ1 | mammals | neurons | | (Cheetham et al., 1992) |
| | HLJ1 | mammals | | | (Hoe et al., 1998) |
| | MCG18 | mammals | | | (Silins et al., 1998) |
| | MSJ-1 | mammals | postmeiotic male germ cells | | (Berruti et al., 1998) |
| | MTJ1 | mammals | | | (Brightman et al., 1995) |
| | CSP1 | mammals | synaptic vesicles of neurons | | (Braun and Scheller, 1995) |
| | CSP2 | mammals | synaptic vesicles of neurons | | (Braun and Scheller, 1995) |

solvent (Hartl, 1996). The close proximity of proteins in vivo increases the probability that associations between exposed hydrophobic segments may be intermolecular instead of intramolecular, an event that would lead to aggregation. Hsp70s do not fold proteins per se; rather, they increase the probability that a protein will find its active conformation upon dissociation from the Hsp70 by protecting it from its neighbours.

The Hsp70 reaction cycle was first characterized in *E. coli* and found to be essentially conserved in eukaryotic cells for all cellular functions of Hsp70s (Figure 4)(Szabo et al., 1994). In *E. coli*, the participating factors are DnaK (the Hsp70), DnaJ (the requisite co-chaperone) and GrpE (a general nucleotide exchange factor). The cycle begins with DnaJ binding to the substrate through its zinc finger domain and presenting it to the ATP-bound form of DnaK. The interaction between DnaK and the substrate is weak until DnaJ stimulates ATP hydrolysis, upon which the ADP-bound form of DnaK forms a tight complex with the DnaJ bound substrate. The GrpE nucleotide exchange factor dissociates ADP from the complex. The binding of ATP weakens the affinity of DnaK for the substrate and it is released.

In eukaryotic cells, homologues of DnaJ are capable of stimulating the ATPase activity of Hsp70s, thereby allowing stable binding to substrates (Nagata et al., 1998). There are at least 40 DnaJ homologues in higher eukaryotes (P. Hunter, this study). Recently, the anti-apoptotic factor BAG-1 has been shown to function as a nucleotide exchange factor in the Hsp70 system in mammalian cells (Hohfeld, 1998). The situation in eukaryotes may be more complex with the existance of yet other co-factors. For example, the ADP-bound form of Hsc70 is stabilized by a unique protein called Hip (Hsc70 interacting protein) (Hohfeld et al., 1995). Release of the substrate must be preceded by the dissociation of Hip from the complex so that ADP can be exchanged for ATP.
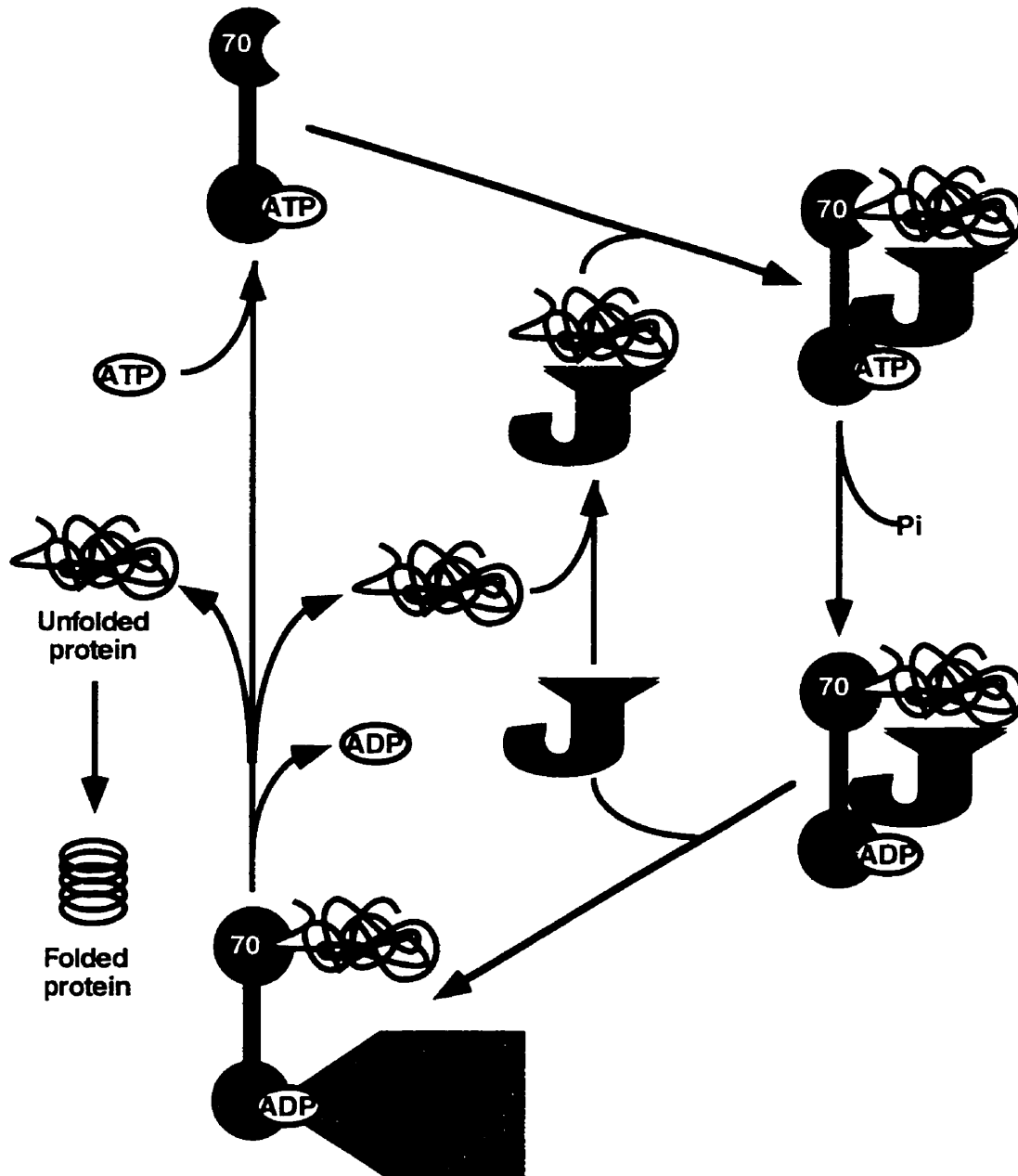
**Figure 4. Hsp70 protein reaction cycle.** Interaction of Hsp70 with a J domain containing protein (**J**) stimulates the ATPase activity of the Hsp70 promoting tight binding to the unfolded protein substrate. A nucleotide exchange factor removes bound ADP enabling the release of the substrate. The interaction between the unfolded protein and the J domain protein has only been demonstrated for a subset of DnaJ-like proteins.

## Structure and Specificity of Hsp70 and DnaJ Homologues

The highly conserved structure of all Hsp70s consists of an N-terminal, 45 kD ATPase domain and a C-terminal, 25 kD peptide binding domain. Small angle X-ray scattering shows that the dumbbell shape of DnaK results in the subdivision of domains (Shi et al., 1996). The crystal structure of the ATPase domain revealed its similarity to actin (Harrison et al., 1997). The peptide binding domain is a novel compact "beta sandwich" linked to an extended structure of alpha helices (Zhu et al., 1996). The hydrophobic peptide binding groove is located in the middle of the beta sandwich. The alpha helices form a lid-like structure with a hinge that is open when the molecule is ATP-bound and which clamps down on the binding site in response to ATP hydrolysis (Zhu et al., 1996).

The site of peptide binding among different Hsp70s is the subject of some debate. The crystal structure of this domain from DnaK suggested that it may be unable to accommodate the preferred amino acid sequence of other Hsp70s (Zhu et al., 1996). This supported the hypothesis that substrate specificity of different Hsp70s is conferred through the peptide binding domain. Further supporting evidence is found from phage display experiments that have shown that each Hsp70 tested bound to specific sequences (Gragerov and Gottesman, 1994). However, evidence against this hypothesis is found in yeast where the cytosolic Hsp70s, Ssa and Ssb, have distinct non-overlapping functions. A chimeric Ssa protien containing the peptide binding domain of Ssb can rescue the Ssa null phenotype. Similarly, an Ssb chimera with an Ssa peptide binding domain rescues the Ssb null phenotype (James et al., 1997). Clearly, in yeast, the specificity of Hsp70s cannot be attributed to peptide binding.

An alternative hypothesis to explain Hsp70 chaperone substrate specificity is that the Hsp70 peptide binding site can accommodate a wide range of hydrophobic sequences and substrate specificity is conferred by the DnaJ-like co-chaperone protein. Homologues

of DnaJ share a highly conserved N-terminal domain and a highly divergent C-terminal domain. The 74 amino acid conserved domain is known as the J domain since it was first characterized in *E. coli* DnaJ. The J domain consists of a scaffolding of two anti-parallel helices aligned along their hydrophobic surfaces (Pellecchia et al., 1996). The loop that connects the helices contains the essential tripeptide HPD; substitutions in this peptide suppress its stimulatory effect on Hsp70 ATPase activity (Wall and Georgopoulos, 1993). Subtle differences in the positioning of HPD in the loop have been shown in yeast to confer specificity in Hsp70-DnaJ pairs (Martin and Hartl, 1997). Based on expressed sequence tags in the NCBI data base, there are at least 40 different proteins in mammals that contain J domains (P. Hunter, this study). Therefore, J domain containing proteins out-number the 11 known mammalian Hsp70s (Tavaria et al., 1996) by a factor of four. This observation combined with the divergence in sequences outside of the J-domain suggests that DnaJ-like proteins act as adaptors conferring substrate specificity to the Hsp70 complex (Silver and Way, 1993).

**Cellular Function of Hsp70**

In eukaryotes there are several genes encoding Hsp70s (Table 1). Different members of the Hsp70 chaperone family have specific roles in a diverse set of cellular processes (Clarke, 1996). Chaperones found in the cytosol act to prevent of aggregation of newly synthesized proteins by binding and protecting hydrophobic segments as they emerge from the ribosome (Nelson et al., 1992). This is thought to be the main activity of the cytosolic Hsp70 (Heat Shock Cognate 70, Hsc70) and the human DnaJ homologue Hdj2 (Nagata et al., 1998). Hsp40, another DnaJ-like protein, was previously thought to participate in this reaction, however, it is unable to associate with newly synthesized proteins in vitro in contrast to Hdj2 (Nagata et al., 1998) Both Hdj2 and Ydj1 (yeast) are the closest relatives of *E. coli* DnaJ, demonstrating similarity to DnaJ in both the J

domain and the C-terminus. They are likely to contain zinc fingers like that of DnaJ, although this structural feature has not been confirmed.

Cytosolic protein folding is often coupled with passage of the protein into the lumen of the endoplasmic reticulum (Lyman and Schekman, 1996). ER lumenal Hsp70s (Kar2p in yeast and Bip in mammals) associate with proteins upon their insertion into the core of the translocase in the ER membrane and cooperate with a J domain containing membrane-bound protein (Sec63p in yeast) (Corsi and Schekman, 1997). Sec63p promotes tight binding of Kar2p to the precursor protein which is then drawn into the ER lumen and released from the chaperone by an unknown mechanism.

Nuclear-encoded mitochondrial proteins are synthesized in the cytosol as precursors and then imported into mitochondria. Protein import requires the function of the mitochondrial Hsp70 (mHsp70) chaperone (Azem et al., 1997; Horst et al., 1997). mHsp70 acts in conjunction with the J domain protein Tim44 (Mdj) which is a subunit of the inner membrane import complex (Horst et al., 1997). The chaperone activity involved in mitochondrial protein import is probably similar to that of ER import in that the precursor protein is pulled into the lumen. The subsequent release of the precursor from mHsp70 is achieved with the assistance of mGrpE, a mitochondrial nucleotide exchange factor (Azem et al., 1997; Horst et al., 1997).

The degradation of proteins in the cell requires a sequestered environment where it can be carried out selectively on proteins which have been targeted for destruction (Baumeister and Lupas, 1997). A complex of proteins known as the proteasome have been discovered in yeast and mammals which targets specific proteins for proteolytic degradation (Baumeister and Lupas, 1997). Hsp70s have been found associated with the entrance to the proteasome and are postulated to function by unfolding protein targets so that they may be threaded as a polypeptide chain into the core of the proteasome (Baumeister and Lupas, 1997). Participating DnaJ homologues and nucleotide exchange factors have not yet been identified.

The proteins of the nuclear matrix are among the most thermal labile proteins in the cell, undergoing denaturation at 43-45 °C (Roti et al., 1997). Heat shock induced protein aggregation in the nuclear matrix is associated with the disruption of nuclear matrix-dependent DNA replication, transcription, hnRNA processing and DNA repair (Roti et al., 1997). Human Hsp70-1 and Hsp70-3 are present in the nucleus and cytosol and their expression uniformly increases in response to heat shock (Roti et al., 1997). Hsp40 (a DnaJ homologue) is normally localized to the cytosol but will move into the nucleus in response to heat shock (Yamane et al., 1995). Hsp40 may function as an adaptor for Hsp70 interaction with nuclear matrix proteins under heat shock conditions (Yamane et al., 1995).

A fraction of all Hsp70-like proteins and co-factors are stress inducible. Stress is thought to be detected by heat shock factors (HSF), transcription factors which bind to the heat shock element (HSE) found in the regulatory sequences of stress inducible genes (Morimoto et al., 1996). Hsp70s participate in their own up-regulation in response to stress by binding a HSF and converting it into a transcriptional activator (Morimoto et al., 1996). Although transcription of many chaperone genes increases in response to heat shock, the repertoire of conditions which can induce their transcription has expanded to include everything that causes "stress" to a cell including viral infection and tumor induction (Morimoto et al., 1996). Hsp70s and other chaperones participate in recovery from heat shock by dissociating heat-induced protein aggregates (Glover and Lindquist, 1998). Mechanisms of recovery from other types of stress are not known.

## Hsp70 relatives: Hsp90

Hsp90 is a close relative of Hsp70, having regions of homology in protein sequence as well as intrinsic ATPase activity (Nadeau et al., 1993). Hsp90 is the specific chaperone of a subset of cellular proteins which includes two broad classes of growth-

regulatory signaling proteins: proto-oncogenic kinases such as erbB2, EGFR, Raf-1 and v-src, and nuclear hormone receptors for androgens, estrogens and glucocorticoids (Stebbins et al., 1997). Hsp90 was discovered as a protein which binds these substrates within a complex which also includes the constitutively expressed Hsc70 and co-chaperones including Hip (Smith et al., 1995). Hsp90 is the target of a family of the antitumor drug, geldanamycin and its derivatives (Whitesell et al., 1994). The antitumor activity of geldanamycin results from its binding to the peptide binding domain of Hsp90. This competitively excludes the proto-oncogenic substrates presumably leaving them unfolded and inactive (Stebbins et al., 1997).

Hsp90 does not bind directly to Hsc70. A 60 kD protein known as Hop (Hsp organizing protein) acts as a molecular bridge through its ability to bind both. Hop is capable of modulating the activity of both chaperones by preferentially binding to the ADP bound form of each (Johnson et al., 1998). In this way, Hop holds Hsp90 in a substrate bound state by competing out the nucleotide exchange factor which would promote substrate turnover (Johnson et al., 1998). This finding is consistent with Hsp90s role in holding proteins in a competent state for activation and/or ligand binding.

**Chaperone Function During Embryonic Development**

Few studies have been done to test the role of chaperones during development. However, the results to date indicate that they can have rather precise functions. Members of Hsp70 chaperone families are some of the first genes to be transcribed following the activation of the zygotic genome in mammals and *Drosophila* (Bendena et al., 1991; Christians et al., 1997). A stress inducible Hsp70 has been found to be specifically upregulated in the mouse placenta at mid gestation (Kothary et al., 1987). Several chaperones have been detected at higher levels in the brain and nervous system compared to other tissues (Loones et al., 1997). These include stress-inducible Hsp70 and

constitutively expressed Hsc70 and Hsp90β. The function of Hsp70s depends on co-chaperone proteins, yet the expression of only a few have been studied in any detail. Two isoforms of a human DnaJ homologue, Hsj1a and Hsj1α, are expressed in the human brain (Cheetham et al., 1992). A subgroup of DnaJ homologues, the cysteine string proteins, are expressed exclusively in neurons in both *Drosophila* and mammals (Ganetzky, 1994). While the preponderance of chaperones in neural tissue is interesting, its biological significance remains unknown.

Another organ with interesting chaperone expression is the testis. Five different members of the Hsp70 family are expressed in the mouse testis and two of these are testis-specific (Eddy, 1995). Hsp70t expression appears in the testis at puberty and is localized to post-meiotic germ cells (Dix et al., 1996). Hsp70-2 is specifically upregulated during testis development in utero and becomes localized to the synaptonemal complexes of late prophase spermatocytes during germ cell maturation (Dix et al., 1996). Targeted mutation of Hsp70-2 in mice resulted in failed spermatogenesis and widespread apoptosis of the germ cells (Dix et al., 1996). These defects were associated with failure of cdc2 to bind to cyclin B1 in a complex which is necessary for the completion of meiosis I. The addition of recombinant Hsp70-2 protein to testis extracts from Hsp70-2 null mice restored cdc2/cyclin B1 association and activity (Zhu et al., 1997). HSP70-2 binds to cdc2 but not cyclin B1. This suggests that HSP70-2 could be acting as a specific chaperone for cdc2 in the testis. Because cdc2 functions in female germ cell meiosis and during mitosis in somatic cells, it raises the question of why HSP70-2 is specifically required for cdc2 activity in the testis but not in any other cell type? It could be that other Hsp70s fill this role in other cell types. Alternatively, cdc2 regulation during meiosis in male germ cells may be unique.

None of the 40 or more DnaJ homologues have been mutated in mammals (with the exception of MRJ, the subject of this thesis). However, in a screen for cell proliferation defects in *Drosophila*, one of the identified genes, *lethal (2) tumorous*

*imaginal discs*, was found to encode a protein which contains an N-terminal J domain but is otherwise unique (Kurzik-Dumke et al., 1995). Interestingly, none of the other genes that when mutated, produced tumor-like phenotypes were known tumor suppressors! The most severe mutations in *lethal(2)tid* result in larvae that die upon formation of the puparium with imaginal disc cells which have overproliferated and failed to differentiate (Kurzik-Dumke et al., 1995). The mechanism by which the product of *l(2)tid* participates in imaginal disc cell cycle regulation is under investigation.

# CHAPTER TWO

## *Mrj* ENCODES A DNAJ-RELATED CO-CHAPERONE THAT IS ESSENTIAL FOR MURINE PLACENTAL DEVELOPMENT

The data in this chapter, except for Figure 7, have been submitted for publication with the following authors: Patricia J. Hunter, Bradley J. Swanson, Melissa A. Haendel, Gary E. Lyons, and James C. Cross. The studies in this chapter were performed by the author except: (1) The immunostaining data shown in Figures 12 and 13 were performed by James Cross. (2) The *Mrj* mRNA in situ hybridization studies shown in Figure 5 were performed by Bradley Swanson. (3) The isolation of the 6AD1 cell line, 5' RACE and cDNA cloning were performed by Gary Lyons, Bradley Swanson and Melissa Haendel.

**Introduction**

Implantation and formation of the placenta are critical for embryonic survival in eutherian mammals. Indeed much of early embryonic development is devoted to establishing extraembryonic cell types which make up the placenta (Copp, 1995; Cross et al., 1994; Rossant, 1995). The early events of placentation involve formation of the trophoblast lineage, the epithelial component of the placenta which attaches the conceptus to the uterus. A critical point in gestation occurs when simple diffusion of gases and nutrients from the mother is no longer sufficient to maintain embryo viability and a transformation in placental structure must occur (Copp, 1995; Cross et al., 1994). In the mouse, this occurs at mid-gestation with the formation of the labyrinth, a vascularized placenta. The labyrinth is a 'chorioallantoic placenta' in that it forms after attachment of the mesodermally-derived allantois to trophoblast cells of the chorionic plate (chorioallantoic fusion). Thereafter, extensive morphogenesis produces the three-dimensional labyrinthine structure which consists of narrow maternal blood sinuses lined by trophoblast cells. In this way trophoblast cells act as a barrier between the maternal and fetal blood compartments.

A number of genes that are essential for early development and morphogenesis of the placenta have been identified (Copp, 1995; Cross et al., 1994; Rinkenberger et al., 1997). These include transcription factor genes that are essential for formation and/or maintenance of different trophoblast cell subtypes; *Err2* of the chorion (Luo et al., 1997), *Mash2* of the spongiotrophoblast (Guillemot et al., 1994; Tanaka et al., 1997) and *Hand1* (formerly called *Hxt/eHAND*) of trophoblast giant cells (Riley et al., 1998). *Err2* mouse mutants fail to form a chorioallantoic placenta because they lack chorionic trophoblast cells. Lack of a chorioallantoic placenta can also be due to primary defects in allantoic outgrowth, caused by mutations in genes such as *brachyury* (Gluecksohn-Schoenheimer, 1944), DNA methyltransferase (Li et al., 1992), *Lim1* (Shawlot and Behringer, 1995) and

*Cskl* (Thomas et al., 1995). The attachment of the allantois to the chorion depends on specific cell adhesion molecules. Vascular cell adhesion molecule-1 (VCAM1) is expressed on the distal tip of the allantois in anticipation of binding to its receptor, α4 integrin, which is expressed on the surface of the chorion (Gurtner et al., 1995; Kwee et al., 1995; Yang et al., 1995). Deficiencies in either VCAM1 or α4 integrin result in failure of chorioallantoic fusion in mice (Gurtner et al., 1995; Kwee et al., 1995; Yang et al., 1995). However, this phenotype occurs in only a portion of mutant conceptuses indicating that the VCAM1/α4 integrin interaction is not the only mechanism mediating chorioallantoic fusion.

One approach that has been widely used to identify new developmentally important genes is gene trapping in murine embryonic stem (ES) cells. We have made use of the ROSAβgeo retroviral vector (Friedrich and Soriano, 1991) which contains a promoterless *βgeo* gene, a fusion of β-galactosidase and neomycin resistance genes, flanked by a splice acceptor at the 5' end and a polyadenylation signal at the 3' end. If the *βgeo* cassette inserts into a transcriptionally active gene, the βgeo protein will be expressed, thus conferring neomycin resistance. In addition, the expression pattern of the trapped gene can be observed by staining specimens for β-galactosidase activity. In about 30% of the cases, the vector insertion disrupts gene function thus producing a mutant phenotype (Friedrich and Soriano, 1991). In our screen, expression patterns of "trapped" genes were studied by in situ hybridization using probes from endogenous sequences that were cloned by 5' RACE (Baker et al., 1997). One ES cell line (6AD1) was selected for further study based on its early expression pattern. Subsequent analysis revealed that this line carries the *βgeo* insertion in a novel gene, named *Mrj* (*m*ammalian *r*elative of Dna*J*), that we show here is essential for chorioallantoic fusion. DnaJ-related proteins in other organisms function as adaptors and activators for HSP70-type chaperones (Hartl, 1996). There are at least 40 DnaJ-related proteins in mammals whose precise molecular and cellular functions are largely unknown. The specific nature of the *Mrj* mutant phenotype

described in this study suggests that these proteins may not have overlapping functions and thus highlights a specificity in their functions.

**Materials and Methods**

### Cloning of the *Mrj* gene

The 6AD1 cell line was identified in a previously described gene trap screen (Baker et al., 1997) using the ROSAβgeo retrovirus (Friedrich and Soriano, 1991) to infect R1 embryonic stem (ES) cells. The allele created by the proviral integration was called *6AD1βgeo*. The 6AD1 cell line was propagated with or without STO feeder cells (ATCC). The medium consisted of 15% fetal bovine serum (HyClone), 0.1 mM β-mercaptoethanol, 2 mM L-glutamine, 0.1 mM MEM nonessential amino acids, 150 μg/ml G418 and 0.1% (v/v) leukemia inhibitory factor in DMEM. mRNA was harvested from the 6AD1 cell line and 5' rapid amplification of cDNA ends (RACE) was performed as previously described (Baker et al., 1997) in order to clone cDNA adjacent to the *βgeo* insertion. A 90 bp 5' RACE product, which represented the 5' end of the *Mrj* cDNA, was cloned and used to screen a λgt10 cDNA library made from E8.5 embryos (kindly provided by Dr. Brigid Hogan). A cDNA of approximately 1.6 kb was recovered and was cloned as two EcoRI fragments of approximately 400 (pC400) and 1200 bp (pC1200) encompassing the entire mRNA. cDNA fragments were ligated into pBluescript (KS-) (Stratagene) and sequenced. The full length cDNA sequence was deposited in GenBank (accession no. AF035962).

To clone genomic DNA 5' to the *βgeo* insertion site (from intron one), inverse PCR was performed as previously described (Jonsson et al., 1996) using primers, oriented in divergent directions, which anneal to sequences in the 3' LTR (5'-TGGGAGGGTCTCCTCTGAGT-3') and β-galactosidase (5'-CACATGGCTGAATATC GACGGTT-3') regions of the *βgeo* insertion. To prepare the template DNA, genomic

DNA isolated from 6AD1 cells was digested with *Eco*RI, diluted, ligated to form circular DNAs and then linearized with *Eco*RV. A single band of approximately 700 bp was produced after PCR amplification. It was ligated into pBluescript to produce the plasmid pE5G and sequenced. Southern blots made from 6AD1 cell genomic DNA confirmed linkage between the cloned intronic DNA and *βgeo*.

## ES cell aggregation and mouse breeding

Aggregation chimeras were generated with 6AD1 ES cells using wild-type CD-1 morulae as previously described (Nagy and Rossant, 1993). Two founder male chimeras were backcrossed to wild-type 129Sv and outcrossed to CD-1 females to produce progeny that were heterozygous for the *6AD1βgeo* allele. Heterozygous mice were intercrossed to produce homozygotes.

## Southern and northern blot hybridization

Southern blot analysis of genomic DNA isolated from tail samples, yolk sacs or whole embryos (Riley et al., 1998) and northern blot analysis of tissue total RNA (Cross et al., 1995) were performed as previously described. Since the *Mrj* coding region is similar to several other *DnaJ*-related genes in mice, we generated a 3' untranslated region probe that was *Mrj*-specific. The pC1200 plasmid containing part of the *Mrj* cDNA was digested with *Eco*O109I and re-closed to produce the plasmid pE3 which contained only the distal 3' untranslated region. The *Asp*718/*Hind*III fragment of pE3 was used as the probe. The *Xho*I/*Eco*RV fragment of pSAβgeo (Friedrich and Soriano, 1991) was used as a probe to detect *βgeo* sequences. The *Eco*RI fragment of pE5G (genomic sequence from intron one) was used as a probe to detect by Southern blot analysis a polymorphism in the *Mrj* locus caused by the insertion of *βgeo* (see Figure 6).

## Conceptus dissections and x-gal staining

Conceptuses were dissected at various gestational ages: noon of the day that a vaginal plug was detected was defined as E0.5. For routine histology, conceptuses were fixed in 4% paraformaldehyde and paraffin embedded. For X-gal staining, specimens were fixed for 15 to 30 minutes in 1% formaldehyde, 0.2% glutaraldehyde, 0.02% NP-40, 5 mM EGTA, 2 mM $MgCl_2$, 0.1 M sodium phosphate (pH 7.3). Specimens were stained whole, or as cryosections, for 4-24 hours at 37°C in 0.1% 4-chloro-5-bromo-3-indolyl-β-D-galactopyranoside (X-gal, Nova Biochem), 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$ in buffer (0.02% NP-40, 0.01% deoxycholate, 2 mM $MgCl_2$, 0.1M sodium phosphate (pH 7.3). For cryosections, fixed conceptuses were equilibrated in 15% followed by 30% sucrose in PBS for 12 hours each at 4°C. Conceptuses were then embedded in OCT medium (Miles) and stored at -80°C prior to cutting into 10 μm sections with a cryostat. Following X-gal staining, sections were counterstained with eosin (Sigma).

## In situ hybridization

In situ hybridization data presented in Figure 5 were prepared as described by Baker et al. (Baker et al., 1997). Otherwise, conceptuses and adult testis were fixed in 4% paraformaldehyde, 0.02% glutaraldehyde in PBS and paraffin embedded. Histological sections (5 μm) were either stained with Harris' haematoxylin and eosin (Sigma) or subjected to in situ hybridization (Millen and Hui, 1996). Antisense (^{33}P)-labeled riboprobes were prepared using an RNA transcription kit (Stratagene). Probes specific to *Gcm1* (Altshuller et al., 1996), *Err2* (Pettersson et al., 1996), *4311* (Lescisin et al., 1988), and *Pl1* (Jackson et al., 1986) have been previously described. A *Mrj*-specific riboprobe was prepared from pE3 which was linearized with *Asp*718. A *βgeo* riboprobe was prepared from pSAβgeo which was linearized with *Pst*I. After development, the sections were counterstained with Carazzi's haematoxylin.

**Immunohistochemistry**

Conceptuses were fixed in 2% paraformaldehyde for 2 hours at 4°C, equilibrated in 8% followed by 18% sucrose in PBS for 4 and 12 hours, respectively, at 4°C and finally embedded in OCT medium (Miles) and stored at -80°C. Cryosections were air-dried and post-fixed in acetone at -20 °C for 5 minutes. They were then subjected to immunoperoxidase staining for VCAM1 using the MK-2 monoclonal antibody (Gurtner et al., 1995) (generously provided by Dr. Myron Cybulsky), α4 integrin using the PS-2 monoclonal antibody (Yang et al., 1995) (Chemicon), and E-cadherin using the DECMA-1 monoclonal antibody (Sigma). Horseradish peroxidase-conjugated secondary antibodies (Amersham) were used at a 1:50 dilution.

**Results**

**Identification and expression of the *Mrj* gene**

A gene trap screen was previously performed by infecting R1 ES cells with the ROSAβgeo retrovirus vector (Baker et al., 1997). Infected ES cells were selected for resistance to G418 and β-galactosidase expression. Sequence 5' to the *βgeo* insertion was cloned by rapid amplification of cDNA ends (RACE) using mRNA isolated from the ES cells. The cDNAs were subsequently used to generate riboprobes for analyzing mRNA expression patterns using in situ hybridization. The 6AD1 ES cell line, from which the *Mrj* gene was identified, was selected for further study because of interesting early expression in trophoblast giant cells.

The expression of *Mrj* during mouse development was studied in detail by following β-galactosidase expression in conceptuses carrying the *6AD1βgeo* allele. Mice carrying this allele were produced by first generating chimeras from the 6AD1 ES cells. The chimeric males were bred to wild-type females in order to produce progeny that were heterozygous for the *6AD1βgeo* allele (*+/6AD1βgeo*). Conceptuses were dissected at

embryonic days (E) 7.5 to 15.5 and stained with X-gal to detect β-galactosidase activity. β-galactosidase activity was evident in the embryo proper at E7.5 at all stages thereafter (Figure 5A-C), though some tissues demonstrated notably higher activity. Subsequent in situ hybridization experiments revealed that the β-galactosidase activity in the embryo faithfully replicated the pattern of the wild-type *Mrj* gene (Figure 5, compare D vs. E, G vs. H). Specifically, *Mrj* expression was detected in the ganglion neural layer of the developing retina (Figure 5C,F). Beginning at E12.5, *Mrj* expression took on a restricted pattern in the brain with consistently high expression in the trigeminal ganglia, diencephalon and midbrain (Figure 5G,H). Other prominently expressing embryonic tissues included the dorsal root ganglia (Figure 5D,E), thymus (Figure 5D,E), nasal epithelium (Figure 5G,H) and testis (not shown).

Expression of *Mrj* and *βgeo* in adult organs of *+/6AD1βgeo* mice was assessed by northern blot analysis. *Mrj* mRNA was readily detected in the testis, uterus, liver and brain with somewhat weaker expression in the eye, heart and gut. The mRNA was not detected in muscle or kidney (Figure 6). The *βgeo* transcript showed a similar tissue distribution except that expression was not detected in the eye and heart.

Expression of *βgeo* in the testis of adult *+/6AD1βgeo* mice was further localized within the seminiferous tubules. β-galactosidase activity was detected throughout the tubules at levels which fluctuated in a periodic pattern along the length of the tubule (Figure 7A). In cross-sections, adjacent tubules could be seen expressing different levels of *βgeo* mRNA which localized to the germ cells and not Leydig cells, Sertoli cells, blood vessels or mature sperm (Figure 7B,C).

**Mrj is expressed in the trophoblast lineage of the placenta**

At all embryonic stages examined, the highest β-galactosidase activity in the entire conceptus was observed in trophoblast giant cells of the placenta (Figure 8A,C). Slightly lower activity was detected in secondary giant cells (which form around the ectoplacental

**Figure 5. Expression of *Mrj* during embryonic development.**

X-gal staining of heterozygous embryos (*+/6AD1βgeo*) (A-D, G) and in situ hybridization on sections of wild-type embryos (E, F, H). (A) E7.5. (B) E8.5. (C) E12.5. (D) E15.5. Embryo was cut in the coronal plane at the C6 vertebra to reveal high expression in the thymus and dorsal root ganglia. (E) E17.5 embryo section from mid-C6 vertebra. (F) Coronal section through an E15.5 embryonic head. N.B. the pigmented layer of the retina is refractile in darkfield illumination and does not represent hybridization signal. (G) Mid-sagittal view of E15.5 embryo to reveal β-galactosidase activity in brain and nasal epithelium. (H) E15.5 sagittal section. de, diencephalon; drg, dorsal root ganglia; mb, midbrain; nc, neopallial cortex; ne, nasal epithelium; rn, ganglion layer of neural retina; th, thymus; tgg, trigeminal ganglia; s, somites.
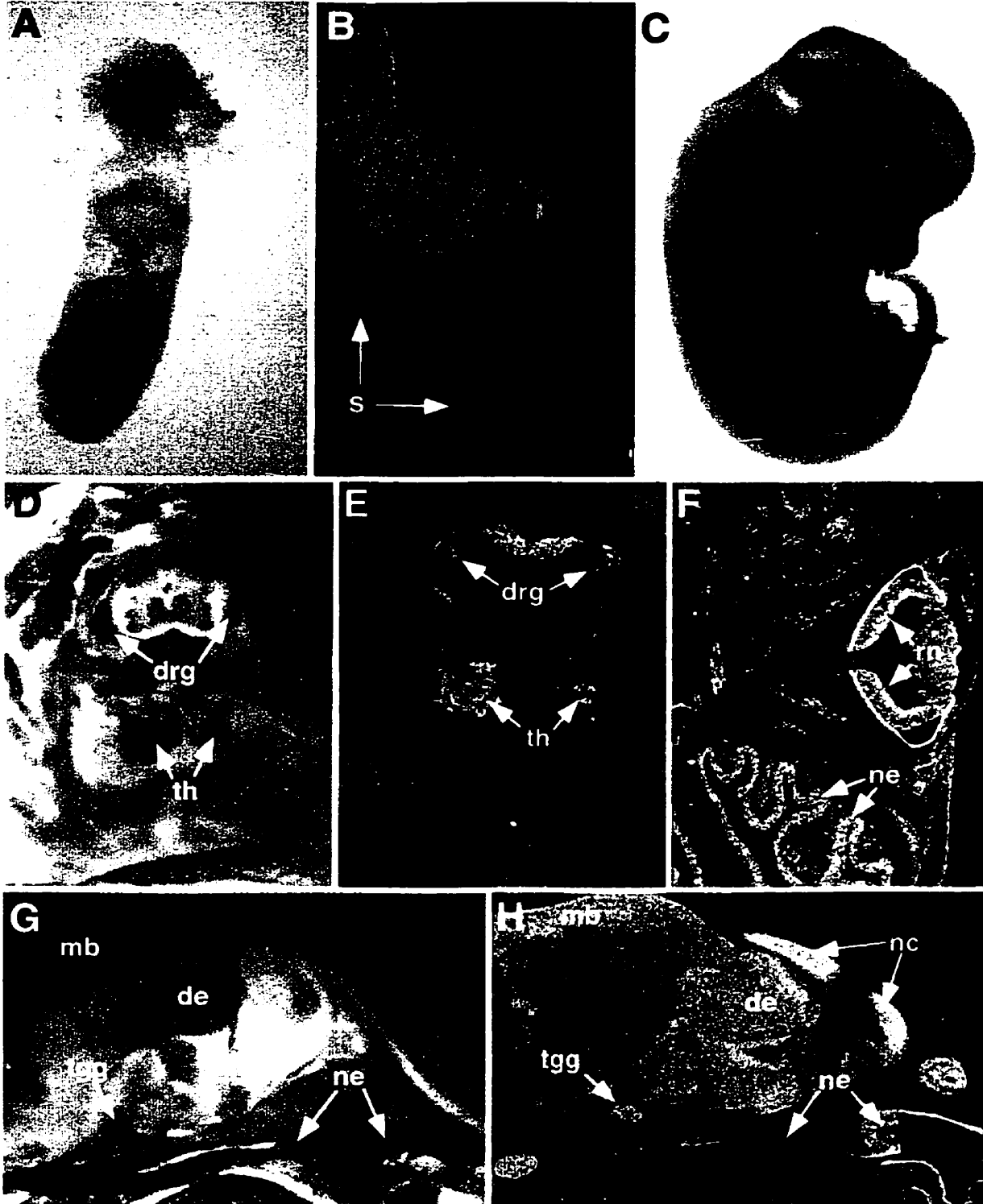
**Figure 6. Expression of *Mrj* in adult mouse tissues.**

Total RNA (10 µg) harvested from organs of *+/6AD1βgeo* mice was used to make two equally loaded blots. The blots were probed with *βgeo* and the *Mrj* 3' untranslated region probes. In, intestine; Ki, kidney; Br, brain; Li, liver; He, heart; Mu, muscle; Ut, uterus; Te, testis; Ey, eye.
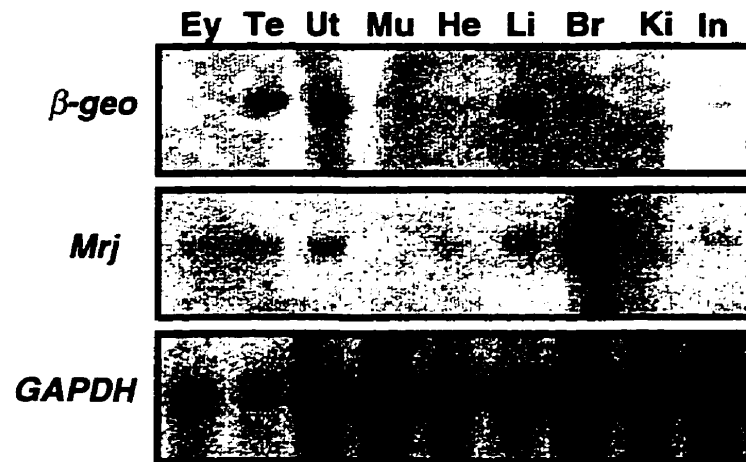
**Figure 7. Expression of *Mrj* in adult mouse testis.**

X-gal staining (A) and in situ hybridization (B,C) of the seminiferous tubules of *+/6AD1βgeo* male mice. (A) Whole tubule showing banding pattern of high β-galactosidase activity. (B) Darkfield cross-section of two tubules expressing *βgeo* mRNA at different levels. (C) Expression is localized to germ cells and not Leydig cells, Sertoli cells or mature sperm on the lumen of the tubule. gc, germ cell; lc, Leydig cell; sc, Sertoli cell; *, lumen.
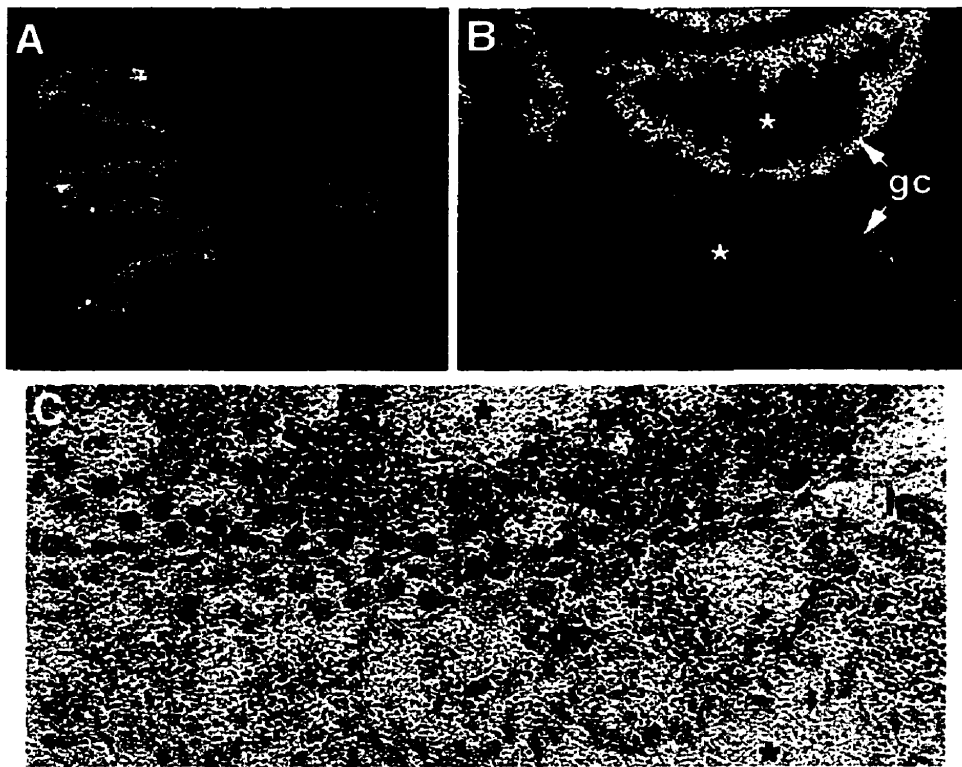
**Figure 8. *Mrj* expression in the placenta.**

Heterozygotes (*+/6AD1βgeo*) x-gal stained (A-C) and in situ hybridization (D,E). (A) Implantation site (embryo removed) showing β-galactosidase activity in giant cells (arrowheads) and the chorion but not the ectoplacental cone. (B) Embryo with yolk sac, chorion and ectoplacental cone attached. Note that β-galactosidase activity is not present in the allantois. (C) Section of a mature placenta at E12.5. Arrowheads indicate giant cells. The dotted line demarcates the border between the spongiotrophoblast layer and the labyrinth. (D,E) Comparison of *Mrj* (D) to *βgeo* (E) mRNA expression in serial histological sections. Expression of both is detected in the chorion, ectoplacental cone and in giant cells. Arrowheads indicate two giant cells with high expression. epc, ectoplacental cone; ch, chorion; al, allantois; sp, spongiotrophoblast; lab, labyrinth; ec, exocoelomic cavity; dec, decidua. Bar represents 100 μm.

A. epc, ch, E8.5
B. < epc, < ch, < al, E8.5
C.
D. epc, ch, dec, ec, E8.5
E. epc, ch, dec, ec, E8.5
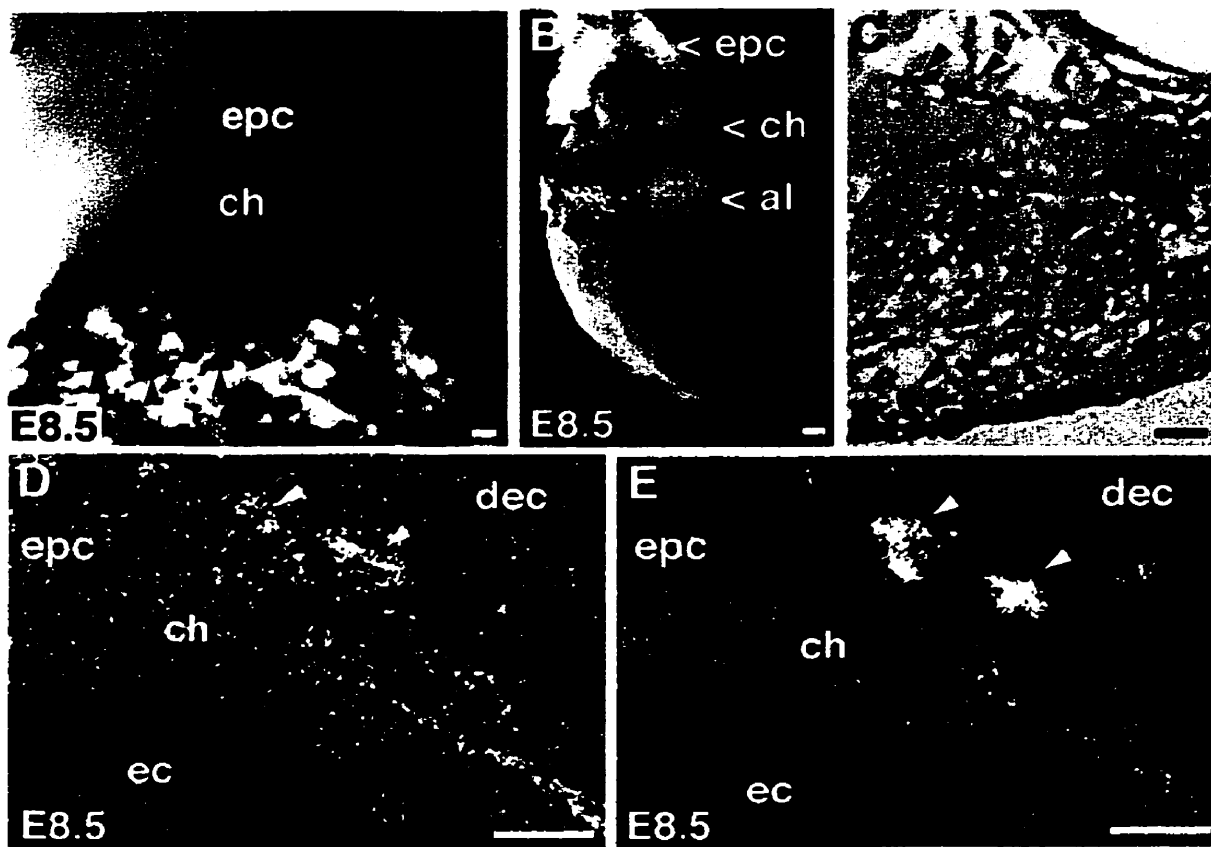
cone) before E9.5. β-galactosidase activity was also evident in trophoblast cells of the chorion but not the ectoplacental cone at E8.5, and in the labyrinth but not spongio-trophoblast at E10.5 (Figure 8A-C). In situ hybridization was performed on sections from placentas of similar stages. *Mrj* and *βgeo* mRNAs were detectable in trophoblast cells of the chorion and ectoplacental cone and in giant cells. However, a subset of giant cells expressed these transcripts at strikingly higher levels (Figure 8D,E). The latter result differed from the β-galactosidase staining which was uniformly high in all giant cells. Another difference between enzyme activity and transcript levels was apparent in the ectoplacental cone and spongiotrophoblast layer. Both *Mrj* and *βgeo* mRNAs were detected by in situ hybridization whereas β-galactosidase activity was never observed (Figure 8A-C). *Mrj* expression was never detected in the allantois, which provides the mesodermal component of the placenta; this tissue was negative for β-galactosidase activity as well as *Mrj* and *βgeo* mRNAs (Figure 8B). After chorioallantoic fusion and subsequent formation of the labyrinth, β-galactosidase activity was detected in the trophoblast component of the labyrinth (Figure 8C), a pattern which resembled the *Mrj* mRNA expression (data not shown).

## *Mrj* encodes a novel member of a large family of DnaJ-related proteins

The *Mrj* cDNA was cloned from an E8.5 mouse embryo cDNA library using the RACE product as the initial probe. A cDNA was recovered which represented 1.6 kb of sequence in total. This size was similar to the predicted size of the full length mRNA based on northern blot analysis of mouse placental mRNA (Figure 10D). The sequence of the *Mrj* cDNA predicted an open reading frame encoding a 242-amino acid protein (Figure 9A). Several cDNAs were identified in the NCBI database of human expressed sequence tags (ESTs) that together represent the complete human *Mrj* cDNA. The open reading frames of the mouse and human cDNAs were 96% and 90% identical in nucleotide and amino acid sequence, respectively (Figure 9A).

**Figure 9. Sequence comparison of MRJ with other mammalian DnaJ-related proteins.**

(A) Amino acid sequence alignment of human and mouse MRJ protein with MSJ1, HSJ1 and HSP40. Amino acids identical to human MRJ are in bold. The gaps in sequence were introduced in order to maximize the sequence alignments. The J domain and three other regions of similarity are shown. H.s., *Homo sapiens* ; M.m., *Mus musculus*. (B) Evolutionary tree analysis of mouse DNA sequences from GenBank and dbEST showing divergence of sequences encoding J domains. Tree was generated using the Multi-alignment/Evolutionary tree software (UPGMA method) of Genetyx Co., Japan.

**A**

```
M.m. MRJ   M VDYYEVLGVQRHASPEDIKKAYRKQALKWHPDKNPKNKEEAIRKFKQVAEAYEVLSDAKKRDIYDKYGKEGLNGGG GGGIHFD  SP
H.s. MRJ   M VDYYEVLGVQRHASPEDIKKAYRKLALKWHPDKNPKNKEEAIRKFKQVAEAYEVLSDAKKRDIYDKYGKEGLNGGG GGGSHFD  SP
M.m. MSJ-1 M VDYYEVLGVPRQASAEAIRKAYRKLALKWHPDKNPKHKEEAIRKFKQVAQAYEVLSDV KREVYDRCGEVGEVGGGGAAGSPFHDA
H.s. HSJ1  M ASYYEILDVPRSASADDIKKAYRRKALQWHPDKNPDNKEFAEKKFKEVAEAYEVLSDKHKREIYDRYGRERLTGTG TGPSRAEAGSGG
H.s. HSP40 MGKDYYQTLGLARGASDEEIKRAYRRQALRYHPDKNKE   PGAEEKFKEIAEAYDVLSDPRKREIFDRYGEEGLKGSGPSGGSGGGANGTS
          _____
                           Region I   J Domain                                          Region II
M.m. MRJ   FEFGFTFRNPDDVFREFFGGRDPFSFDFFE DPFDDFFGNRRGPRGNRSRGAAPFFSTFSGFPSFGSGFPAFDTGFTPFGSLGHGGLTSFS
H.s. MRJ   FEFGFTFRNPDDVFREFFGGRDPFSFDFFE DPFEDFFGNRRGPRGSRSRGTGSFFSAFSGFPSFGSGFSSFDTGFTSFGSLGHGGLTSFS
M.m. MSJ-1 FQYVFSFRDPAEVFREFFGGHDPFSFDFFGGDPLENFFGDRRSTRGSRSRGAVPFSTSFTEFPGFGGGFASLDTGFTSFGSPGNSGLSSFS
H.s. HSJ1  PGFTFTFRSPEEVFREFFGSGDPFAELFDLG PFSELQ NR   GSRHS  GPFFTFSSSFP            GH    SDFS
H.s. HSP40 FSTYFHGD PHAMFAEFFGGRNPFDTFFGQRNGEEGMDIDDPFS GFPMGMGGFTNVNFGRSRSAQEPARKKQDPPVTHDLRVSLEEIYSG
          _____
              Region III
M.m. MRJ   STSFGGS GMGNFKSISTSTKIVNGKKITTKRIVENGQERVEVEEDGQLKPLTINGKEHLLRLDNK
H.s. MRJ   STSFGGS GMGNFKSISTSTKMVNGRKITTKRIVENGQERVEVEEDGQLKSGTINGKEQLLRLDNK
M.m. MSJ-1 M SCGGGAA GMYKSVSTSTEIINGKKITTKRIVENGQERVEVEEDGELKSLIINGREQLLRINTQ
H.s. HSJ1  SSSFSFSPGAGAFRSVSTSTTFVQGRRITTRRIMENGQERVEVEEDGQLKSVTINGVPDDLARGLE... 133 amino acids
H.s. HSP40 CTKKMKISHKRLNPDGKSIRNEDKILTIEVKKGWKEGTKITFPKEGDQTSNNIPADIVFVLKDKPH... 96 amino acids
          _____
                Region IV
```

**B**



50    60    70    80    90    100
% identity
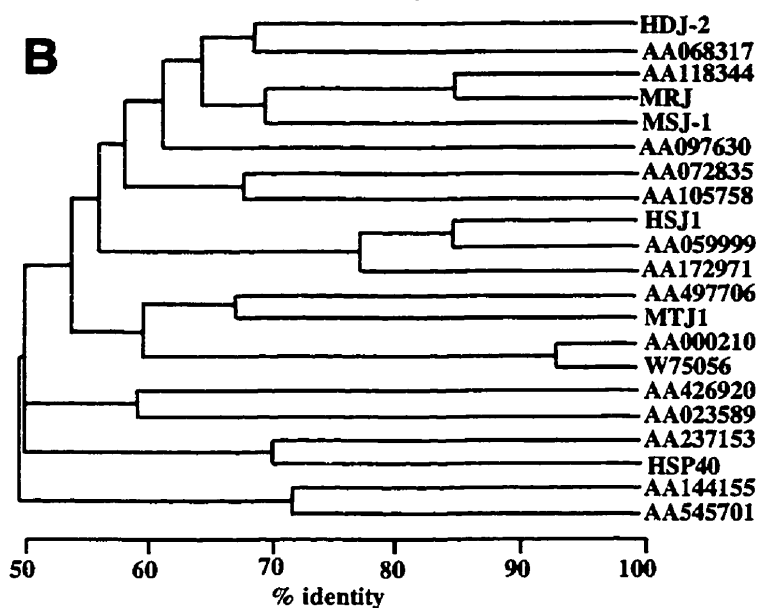
Although the MRJ protein was unique when compared to sequences in GenBank, the N-terminal 74 amino acids were similar to the so-called J domain present in the *E.coli* DnaJ protein, as well as in several proteins in yeast, *Drosophila*, *C.elegans* and mammals. DnaJ-related proteins interact with HSP70 chaperones via the J domain and stimulate their ATPase activity (Hartl, 1996). In searching GenBank and EST databases, we found more than 40 unique cDNA sequences which encode J domain proteins in both humans and mice; each of these sequences was predicted to encode the HPD motif, which is essential for interaction with HSP70, as well as adjacent α-helices which are conserved among family members. Twenty cDNAs encoded J domains which shared greater than 50% amino acid sequence identity with MRJ (Figure 9B shows a dendritogram). Within this group were five previously identified proteins called MSJ1 (Berruti et al., 1998), HSJ1 (Cheetham et al., 1992), HSP40 (HDJ1), (Ohtsuka, 1993; Raabe and Manley, 1991), HDJ2 (Chellaiah et al., 1993) and MTJ1 (Brightman et al., 1995). C-terminal to the J domain, the MRJ protein had three other regions of sequence similarity to three of these family members (Figure 9A, Regions II-IV). Region II is a Gly- or Gly/Phe-rich sequence which is also present in *E. coli* DnaJ. The significance of regions III and IV which are conserved in MRJ, MSJ1, HSJ1 and HSP40 (region III only) is unknown. However, their sequences were unique to these proteins.

## The *βgeo* insertion maps to the first intron of the *Mrj* locus

Southern blot analysis of DNA extracted from 6AD1 cells indicated that a complete copy of *ROSAβgeo* cassette, including full length LTR sequences, had inserted into the *Mrj* locus. Since the fragment of cDNA cloned by RACE represented the first 90 bp of 5' untranslated region in the full length mRNA, this region was assumed to be exon 1. Therefore, *βgeo* had inserted either into exon one or downstream within an intron. To distinguish these possibilities, Southern blot analysis was used to generate a restriction map around exon 1 and the *βgeo* insertion (data summarized in Figure 10A). There was

**Figure 10. The βgeo insertion disrupts the Mrj gene and reduces mRNA expression.**

(A) Schematic representation of the 5' region of the Mrj locus. The line indicates the genomic DNA with restriction enzyme sites indicated. The position of the ROSAβgeo insertion is indicated. Exons, indicated by the lightly shaded rectangles, are not drawn to scale. Bars represent probes for Southern blot analysis. E, EcoRI; EV, EcoRV; H, HindIII; A, Asp718; B, BamHI. (B) Wild-type and 6AD1 ES cell DNA digested with indicated enzymes, Southern blotted and hybridized with probe A. Note that no restriction site polymorphisms are associated with the 6AD1βgeo allele using this probe. (C) DNA from wild-type, 6AD1βgeo heterozygous and homozygous mice digested with EcoRV, Southern blotted and hybridized to probe B (from intron one). (D) Northern blot of total RNA isolated from placentas of wild-type, 6AD1βgeo heterozygous and homozygous conceptuses at E10.5. A Mrj probe representing 3' UTR sequence downstream of the βgeo insertion was used. (E) Bright field (top) and dark field (bottom) views of in situ hybridization using an antisense probe specific to Mrj on sections from a wild-type and a homozygous Mrj mutant embryo at E8.5. Giant cells expressing Mrj are indicated with arrowheads. ch, chorion. Bar represents 100 μm.

**A**

EV

3'LTR | SAβgeo | 5'LTR

2 kb

Exon1

B  H A  B  H EV  A  EV  E  EV

probe A

Exon2
ATG

probe B

**B**

EV | H | A | B

+/+  +/6AD1βgeo  +/+  +/6AD1βgeo  +/+  +/6AD1βgeo  +/+  +/6AD1βgeo

kb
12
5
4
3
2

**C**

+/+  +/6AD1βgeo  6AD1βgeo/6AD1βgeo

kb
12
5
4
3
2

**D**

+/+  +/6AD1βgeo  6AD1βgeo/6AD1βgeo

*Mrj*  < 1.6 kb

18S
rRNA

**E**

+/+  6AD1βgeo/6AD1βgeo

epc
ch

epc
ch
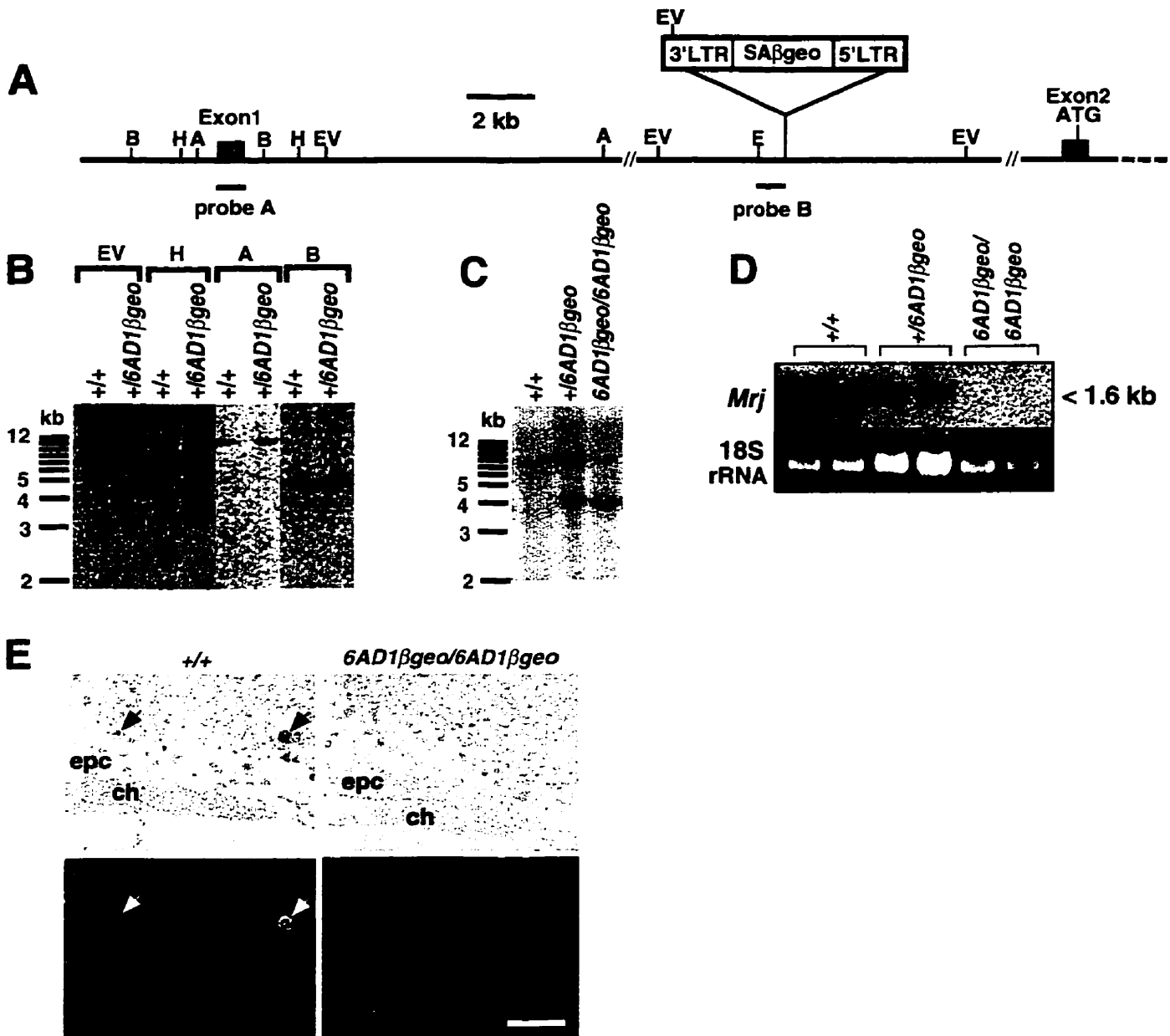
no overlap between the restriction maps around exon 1 and the *βgeo* insertion indicating that the two were separated by some distance (> 12 kb). Because of this distance, we were unable to detect any restriction enzyme polymorphisms on Southern blots caused by the insertion of *βgeo* into the *Mrj* locus when using exon 1 as the probe (Figure 10B). To determine if the insertion had disrupted exon 2 or sequence further 3', we probed Southern blots using distal 3' cDNA probes (plasmids pC400 and pC1200). However, we were unable to detect restriction enzyme polymorphisms (data not shown). We concluded, therefore, that *Mrj* exon sequences were not disrupted by the insertion and that *βgeo* had inserted into intron one. In order to detect polymorphisms associated with the *6AD1βgeo* allele that were required to genotype mice by Southern blotting, we cloned a fragment of genomic DNA flanking the 5' end of *βgeo* by using inverse PCR. The sequence of this fragment was unique and, when used as a probe, revealed restriction site polymorphisms between DNA from wild-type and +*/6AD1βgeo* mice (Figure 10C).

**Disruption of *Mrj* expression from the *6AD1βgeo* allele**

The mapping data indicated that the *βgeo* insertion had not disrupted *Mrj* coding sequence. To determine if the *βgeo* insertion has disrupted the function of the *Mrj* gene, we examined mice carrying the mutant gene for an abnormal phenotype. Heterozygous mice appeared normal and transmitted the *6AD1βgeo* allele at the predicted Mendelian frequency of 50% (Table 2), but in intercrosses of heterozygous animals, no homozygotes were detected among the progeny at birth (Table 2). Progeny from heterozygous matings were then dissected at E8.5 to E14.5. Conceptuses that were homozygous for the *6AD1βgeo* allele were viable only up to about E11.5 (Table 2). The matings summarized in Table 2 represent mice produced by outcrossing the founder chimeras to an outbred background. However, the same phenotype was observed on a 129Sv inbred background.

To investigate the embryonic lethal phenotype of conceptuses that were homozygous for the *6AD1βgeo* allele, we determined if their expression of *Mrj* mRNA

**Table 2. Genotypes of offspring from *6AD1βgeo* heterozygous mice (number of progeny shown)**

| Mating ♂ x ♀ | # litters | +/+ | +/*6AD1βgeo* | *6AD1βgeo/6AD1βgeo* |
|---|---|---|---|---|
| +/*6AD1βgeo* x +/+ | | | | |
| expected | | 50% | 50% | - |
| observed | | | | |
| newborn | 5 | 27 | 26 | - |
| +/+ x +/*6AD1βgeo* | | | | |
| expected | | 50% | 50% | - |
| observed | | | | |
| newborn | 5 | 30 | 28 | - |
| +/*6AD1βgeo* x +/*6AD1βgeo* | | | | |
| expected | | 25% | 50% | 25% |
| observed | | | | |
| E8.5 | 6 | 12 | 25 | 11 |
| E9.5 | 7 | 17 | 46 | 20* |
| E10.5 | 7 | 12 | 43 | 18* |
| E11.5 | 4 | 10 | 20 | 12*$^{\Delta}$ |
| E12.5 | 3 | 4 | 13 | 11$^{\Delta}$ |
| E14.5 | 2 | 6 | 11 | 6$^{\Delta}$ |
| newborn | 7 | 22 | 36 | 0 |

* small embryos, $^{\Delta}$ dead embryos, resorptions

was reduced. Northern blots of E10.5 placental RNA from conceptuses of wild-type (+/+), heterozygous (+/*6AD1βgeo*) and homozygous (*6AD1βgeo/6AD1βgeo*) mutant genotypes were probed with a fragment of *Mrj* gene which lies downstream of the *βgeo* insertion. We were unable to detect any *Mrj* mRNA in homozygous mutant placentas (Figure 10D). Furthermore, *Mrj* transcript expression was significantly reduced (by about 50%) in samples from heterozygous conceptuses. To confirm the northern blot results, mRNA in situ hybridization analysis was performed on histological sections from E8.5 mutant conceptuses using a riboprobe generated from the same 3' fragment. *Mrj* mRNA expression was undetectable in homozygous tissues (Figure 10E). Therefore, the 6AD1βgeo mutation appeared to be a null allele.

## Failure of chorioallantoic fusion in *Mrj* homozygous mutants

The first abnormalities in *Mrj* mutants were apparent at E8.5. At this time, homozygous mutant embryos appeared normal except that chorioallantoic fusion never occurred in the extraembryonic region, in contrast to all wild-type and heterozygous littermates (Table 3; Figure 11). By E9.5, although they had turned, homozygous embryos were smaller than their littermates and arrested at the 18 somite stage. The allantois remained unattached to the chorion (Figure 11). By E11.5, the allantois had formed a loose attachment to the chorion in a few conceptuses. However, most embryos were dead by this stage and placental labyrinth morphogenesis never proceeded. All homozygous mutants were undergoing resorption by E12.5.

We looked for abnormalities in placental histology in homozygous *Mrj* mutants. The allantois appeared normal in mutants except that it remained unattached. All trophoblast cell types were present and were morphologically normal in the homozygous mutant conceptuses at E8.5 (Figure 11B). As development proceeded, the chorionic plate remained intact in the placentas of *Mrj* mutants (Figure 11B) although, starting at E9.5, vacuolated cells and pyknotic nuclei were observed at high magnification. Marker

**Figure 11. Placental phenotype in homozygous *Mrj* mutants.**

(A) Whole mount views of partially dissected feto-placental units at E9.5. Notice that in the heterozygous conceptus, the allantois has attached to the chorion. In homozygous *Mrj* mutants, the allantois does not fuse to the chorion and appears as a bud. (B) Histology of the placenta in *Mrj* mutant conceptuses. Histological sections of wild-type and *Mrj* mutant placentas at E8.5, 9.5 and 10.5. Dotted lines mark the interface between trophoblast giant cells and the decidua. al, allantois; ch, chorion; epc, ectoplacental cone; lab, labyrinth; pl, placenta; sp, spongiotrophoblast; uc, umbilical chord. Bar in panel B represents 100 μm.

**A**  +/6AD1βgeo          6AD1βgeo/6AD1βgeo

pl

< uc

pl

< al

**B**          +/+          6AD1βgeo/6AD1βgeo

E8.5          ch          al          ch          al

E9.5          sp          al

E10.5          lab          ep          ch
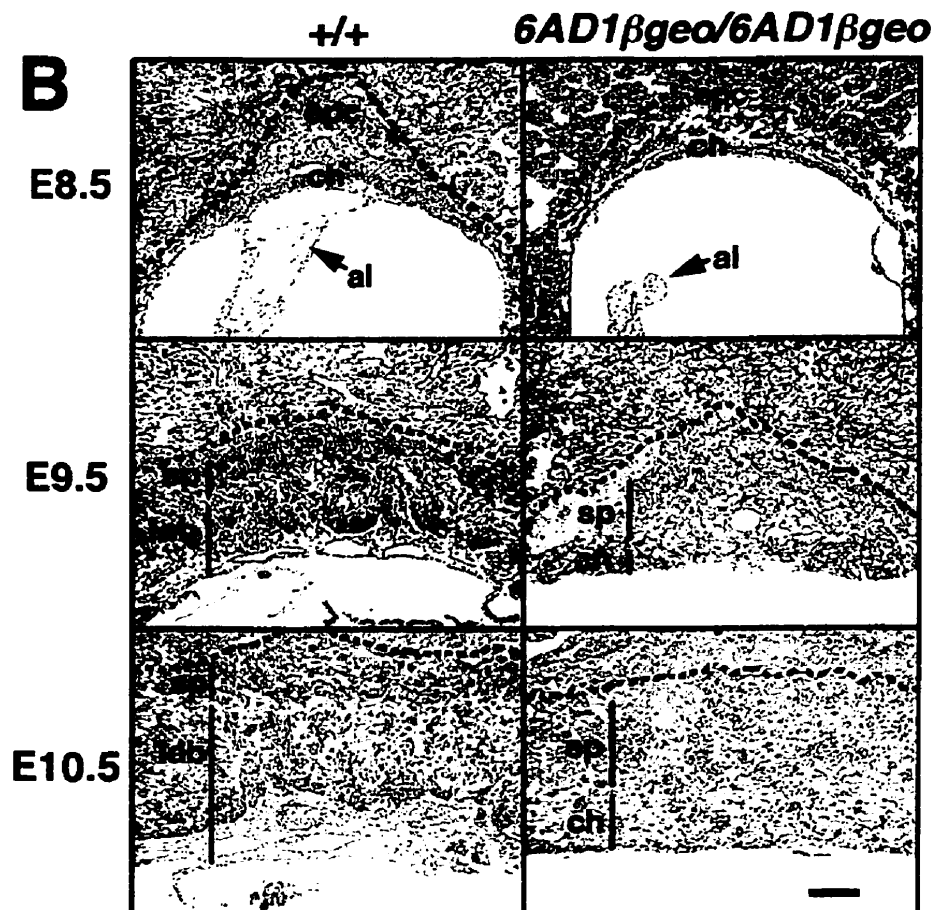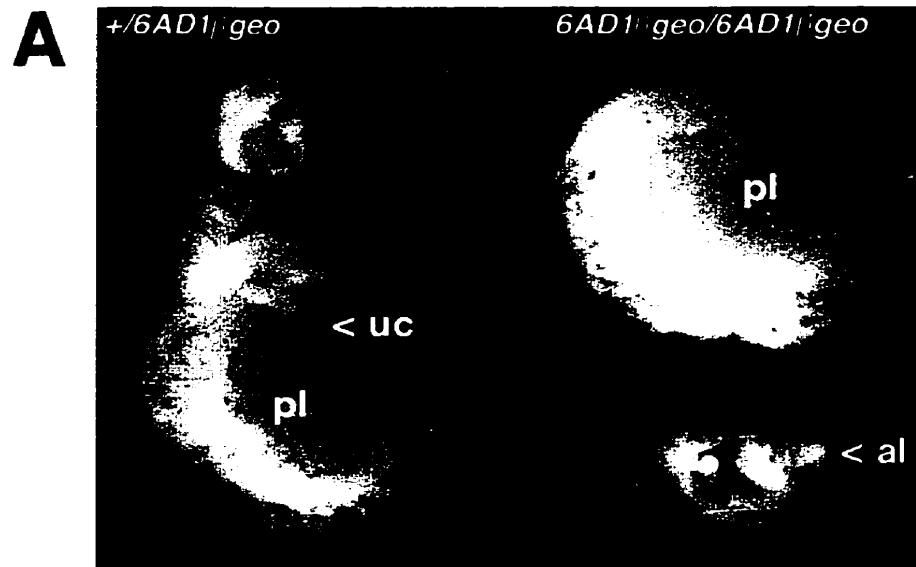
**Table 3. Incidence of placental phenotype in offspring from intercrosses of *6AD1βgeo* heterozygous mice**

| Age | Genotype | Total | Normal | No chorio-allantoic fusion | Labyrinth defects | Resorption |
|-----|----------|-------|--------|----------------------------|-------------------|------------|
| E8.5 | +/+ | 4 | 4 | 0 | - | 0 |
| | +/6AD1βgeo | 12 | 11 | 1 | - | 0 |
| | 6AD1βgeo/6AD1βgeo | 6 | 0 | 6 | - | 0 |
| E9.5 | +/+ | 17 | 17 | 0 | 0 | 0 |
| | +/6AD1βgeo | 46 | 46 | 0 | 0 | 0 |
| | 6AD1βgeo/6AD1βgeo | 20 | 1 | 17 | 2 | 0 |
| E10.5 | +/+ | 8 | 8 | 0 | 0 | 0 |
| | +/6AD1βgeo | 18 | 17 | 0 | 0 | 1 |
| | 6AD1βgeo/6AD1βgeo | 7 | 0 | 6 | 1 | 0 |
| E11.5 | +/+ | 10 | 10 | 0 | 0 | 0 |
| | +/6AD1βgeo | 20 | 20 | 0 | 0 | 0 |
| | 6AD1βgeo/6AD1βgeo | 12 | 0 | 4 | 4 | 4 |

analysis was performed to detect changes in gene expression of trophoblast cell types

(Figure 12). The expression of *Pl1*, a trophoblast giant cell-specific gene (Jackson et al.,

1986), and *4311*, an ectoplacental cone and spongiotrophoblast-specific gene (Lescisin et

al., 1988), were both expressed normally in *Mrj* mutant placentas (Figure 12). We next

examined the expression of chorion-specific markers. E-cadherin is expressed by basal

cells in the chorionic plate prior to allantoic fusion (Reuss et al., 1996) a pattern which

was unaltered in *Mrj* mutants (Figure 12). However, the expression levels of *Err2*

(Pettersson et al., 1996) and *Gcm1* (Altshuller et al., 1996), genes whose expression is

restricted to the chorion at E8.5 and the trophoblast component of the labyrinth at later

stages (Pettersson et al., 1996)( J.C.C., unpublished data), were both significantly reduced

at E10.5. This was particularly evident for *Gcm1* even at E8.5, coincident with the first

observed defects in *Mrj* mutants.


## Normal VCAM1 and α4 integrin expression in *Mrj* mutants

To investigate the molecular basis of the chorioallantoic fusion defect, we looked for

abnormalities in expression of cell adhesion molecules that are known to participate.

Around the time of chorioallantoic fusion (E8.25-8.5), α4 integrin is normally expressed

on the surface of the chorion (Yang et al., 1995) and VCAM1 is expressed on the distal

two thirds of the allantois (Gurtner et al., 1995; Kwee et al., 1995). By immuno-

histochemistry, both of these patterns were observed at E8.5 in *Mrj* homozygous mutants

(Figure 13). During normal development, the timing of receptivity for chorioallantoic

fusion is thought to be tightly regulated (Downs, 1998). It was notable, therefore, that we

saw persistent expression to at least E9.5 of α4 integrin and VCAM1 in *Mrj* mutants in

which chorioallantoic fusion had not occurred (Figure 12).

**Figure 12. Trophoblast marker analysis of homozygous *Mrj* mutants.**

Serial sections of wild-type and mutant (*6AD1βgeo/6AD1βgeo*) conceptuses at E8.5 and 10.5 were probed with antisense riboprobes for *Gcml, 4311, Pl1* and *Err2*, or immunostained to detect E-cadherin. *Pl1* is a marker of trophoblast giant cells. *4311* is a marker of the ectoplacental cone and the spongiotrophoblast layer. *Gcml, Err2* and E-cadherin are expressed in the chorion and labyrinthine trophoblast cells. Note that expression of *Gcml* and *Err2* is reduced in *Mrj* mutant placentas. epc, ectoplacental cone; ch, chorion; sp, spongiotrophoblast; lab, labyrinth. Bar represents 100 μm.

|  | H+E | Gcm1 | 4311 | Pl1 | E-cad |
|---|---|---|---|---|---|

E8.5 +/+

6AD1βgeo/ 6AD1βgeo

|  | H+E | Gcm1 | 4311 | Pl1 | Err2 |
|---|---|---|---|---|---|

E10.5 +/+

6AD1βgeo/ 6AD1βgeo
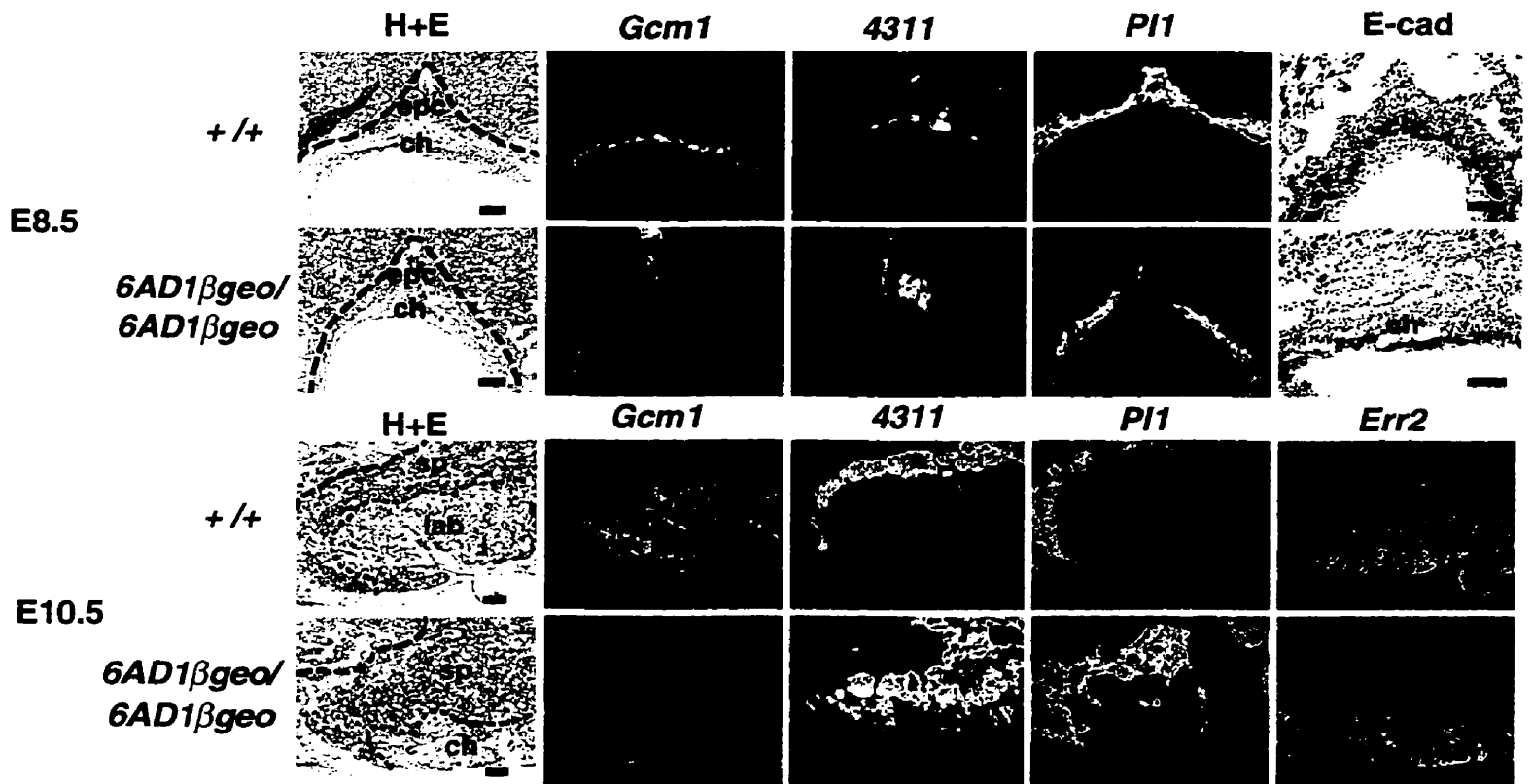
**Figure 13. VCAM1 and α4 integrin expression in the developing chorioallantoic region.** Histological sections of wild-type and homozygous *Mrj* mutant (*6AD1βgeo/6AD1βgeo*) placentas at E8.5 and 9.5 were subjected to immunostaining. Note that both VCAM1 and α4 integrin continue to be expressed in *Mrj* mutants at E9.5 even though fusion between the chorion and the allantois (al) has not occurred. The allantois is not present in the E8.5 mutant conceptus because the embryo was removed for genotyping. Bar represents 100 μm.

**VCAM1**   **α4 integrin**

E8.5

+/+

E8.5

6AD1βgeo/
6AD1βgeo

E9.5

6AD1βgeo/
6AD1βgeo
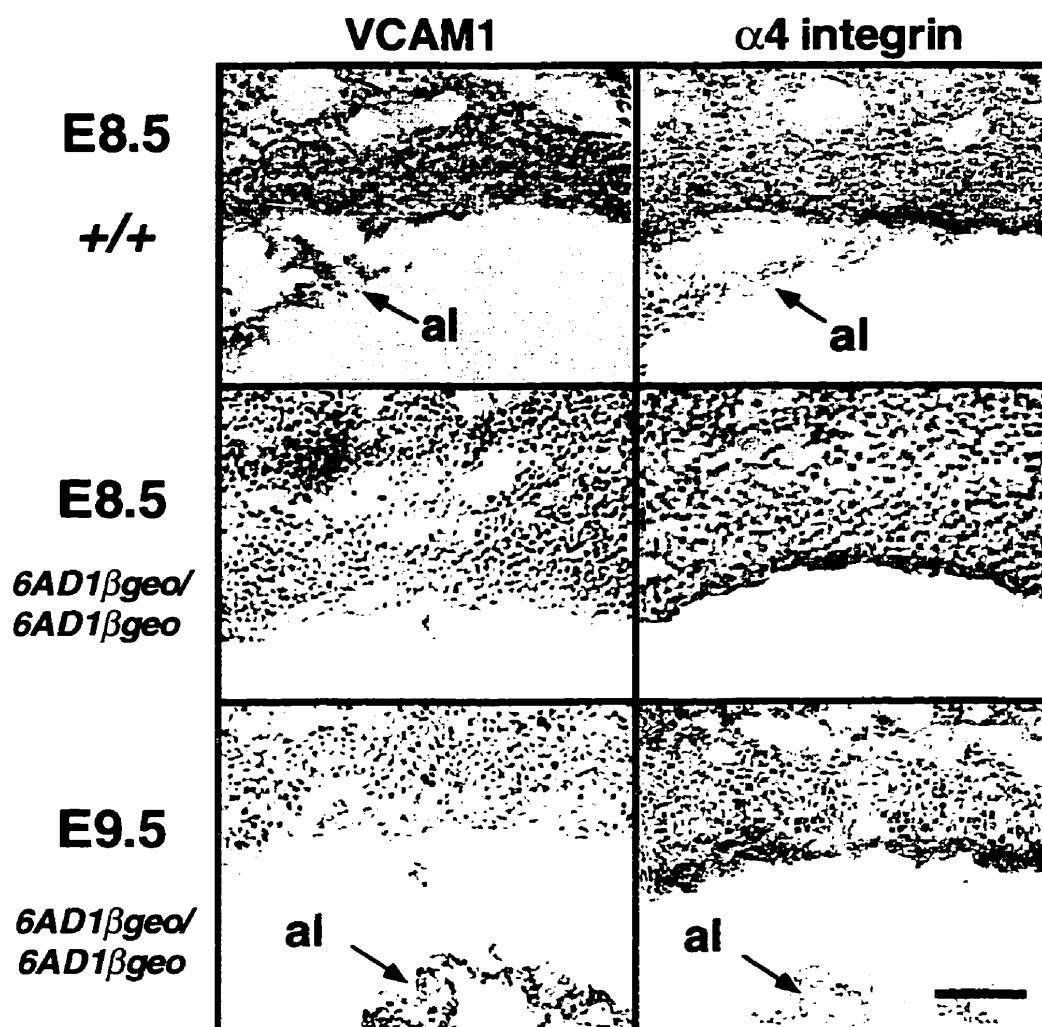
## Discussion

The *Mrj* gene was first identified in the gene trap screen because of its expression in ES cells. However, we have shown that *Mrj* is expressed during development in the placenta and several regions of the embryo, and in some tissues into adulthood. The MRJ protein sequence indicates that it is part of a large DnaJ-related family that contains at least 40 members in mammals. This study is the first to describe the consequences of a mutation in a mammalian DnaJ-related protein. The fact that *Mrj*-deficient conceptuses are unable to complete development suggests that there may be little redundancy of function within this family.

### *Mrj* expression is developmentally regulated

*Mrj* expression occurs broadly in several organs during development and into postnatal life. We only studied in detail its expression during placental development because the phenotype of *Mrj*-deficient conceptuses indicated an early essential function in placental trophoblast development. The trophoblast lineage arises first as the trophectoderm at the blastocyst stage (E3.5 in mice) (Cross et al., 1994; Rossant, 1995). By the early postimplantation period (E6.5-7.5), three anatomically and functionally distinct trophoblast cell types are apparent. Chorionic trophoblasts (also called extraembryonic ectoderm) lie next to the embryo; ectoplacental cone trophoblasts sit as a cap of tissue between the chorion and the outer layer of trophoblast giant cells. Chorionic cells, in addition to contributing to the labyrinth after contact with the allantois, are thought to be the proliferating trophoblast stem cells (Rossant, 1995; Rossant and Ofer, 1977). In culture, chorionic trophoblast cells differentiate first into ectoplacental cone-type and subsequently to trophoblast giant cells (Carney et al., 1993), suggesting that these three cell types represent steps in a differentiation pathway. *Mrj* mRNA is therefore expressed throughout the trophoblast lineage since we detected it in chorion, ectoplacental cone and

giant cells at E7.5 and 8.5. Nonetheless, we have observed a mutant phenotype in only the chorion of *Mrj*-deficient conceptuses.

Expression studies revealed some potentially interesting features of *Mrj* regulation. First, there were differences between *Mrj* and *βgeo* transcript levels in some tissues. For example, *Mrj* transcripts were detectable in the heart and eye, albeit at low levels, but *βgeo* transcripts were not. β-galactosidase expression that appears more restricted than the expression of the endogenous gene has been reported for other gene tapping experiments (Voss et al., 1998). It is possible that the *βgeo* insertion disrupted intronic sequences which regulate tissue-specific transcription or splicing. However, in all other tissues we observed a good correlation between *Mrj* and *βgeo* mRNA expression. Two other interesting features of expression were apparent in the trophoblast lineage. In the trophoblast giant cell population, while expression was detectable in all cells by mRNA in situ hybridization, a much higher expression level was observed in a subset of cells. The same pattern was observed when using the *βgeo* probe. These strongly-expressing cells were randomly distributed around the conceptus in a pattern unlike any other giant cell gene expression pattern that is known to us. Trophoblast giant cells randomly undergo rounds of endoreduplication (MacAuley et al., 1998)(T. Davies and J.C.C., in preparation) and, therefore, these bursts of *Mrj* mRNA expression could be regulated through the cell cycle. Notably, the variable expression level was not apparent from the β-galactosidase staining, which was uniformly strong in every giant cell. An explanation for this difference is that the βgeo protein is stable and, therefore, persists in the cell even though *Mrj* mRNA expression may be variable. Another difference between *Mrj* and *βgeo* mRNA expression and β-galactosidase enzymatic activity was apparent in the spongiotrophoblast layer and its precursor, the ectoplacental cone. β-galactosidase activity was never observed in these trophoblast cells despite the presence of *Mrj* transcripts. Importantly, we detected *βgeo* transcripts in these cells indicating that splicing to the *βgeo* cassette occurred properly. It is unlikely that protein instability

accounts for the absence of β-galactosidase enzymatic activity, since it can be detected in the ectoplacental cone and spongiotrophoblast of ROSA26 conceptuses (Tanaka et al., 1997). It is possible, though, that a *βgeo* transcript with the *Mrj* 5' untranslated region is not efficiently translated in ectoplacental cone or spongiotrophoblast. It will be important to study MRJ protein expression in order to clarify these issues.

## Mrj is essential for chorioallantoic fusion at mid-gestation

Although the *βgeo* insertion in the *6AD1* cell line did not disrupt coding exons, it did create a hypomorphic and perhaps null allele of the *Mrj* gene because we were unable to detect *Mrj* mRNA in conceptuses that were homozygous for the *6AD1βgeo* allele. This likely resulted from failure to splice around the *βgeo* cassette and truncation of the transcript by the polyadenylation signal at the 3' end of the *βgeo* sequence. We also noted that there was a significant reduction in *Mrj* mRNA levels in heterozygotes indicating that there was no compensation for loss of one allele. Despite this, heterozygotes had no obvious placental phenotype and were born at the expected frequency. The adults appeared normal and were fertile but have otherwise not been examined in detail.

*Mrj* is expressed in both the placenta and embryo but we found that the phenotype of *Mrj*-deficient conceptuses can be explained solely by the defect in placentation. The formation of a chorioallantoic placenta is a critical "checkpoint" that must be achieved by mid-gestation (Copp, 1995; Cross et al., 1994). We observed no primary developmental defects of the embryo proper in *Mrj* mutants. The mouse embryo has been reported to continue to develop normally up to the 18 somite stage in culture following the removal of the allantois (Downs and Gardner, 1995). The developmental arrest of the mutant embryos at the 18 somite stage (approximately E9.25) was observed in some mouse mutants which are deficient for VCAM1 (Gurtner et al., 1995; Kwee et al., 1995) and α4 integrin (Yang et al., 1995). Exploring the function of *Mrj* in the embryo proper will thus depend on either developing a tissue-specific knockout or on being able to rescue the

chorion defect. The latter could be accomplished by using the tetraploid chimera technique as has been done with other mutants in which trophoblast function in affected (Riley et al., 1998; Guillemot et al., 1994).

The failure of chorioallantoic fusion can be attributed to defects in chorion trophoblast cell function. First, *Mrj* expression was limited to the chorion and was not detected in the allantois. Furthermore, the allantois was of normal size, showed directed growth and expressed the cell adhesion molecule VCAM1 in *Mrj* mutants. The fact that the chorion formed at the normal time and persisted in *Mrj* mutants implies that the failure probably resulted from a lack of receptivity of the chorion. The mechanism of receptivity which is affected in *Mrj* mutants is unknown since the expression of $\alpha 4$ integrin (which is co-expressed with *Mrj* in the chorion) and VCAM1 was normal. However, because MRJ is likely to function as a co-chaperone it is possible that its deficiency could affect $\alpha 4$ integrin cell surface presentation or function without having an apparent effect on its expression. The immunocytochemistry technique used to observe $\alpha 4$ integrin staining involved the dissolution of cell membranes using acetone. Therefore, it could not distinguish between correctly folded epitopes trapped within the cell and proteins on the surface. It is also important to note that integrin activity can be regulated at the cell surface by 'inside-out signaling' (Ginsberg et al., 1992). Therefore, our data do not preclude the possibility that the failure in chorioallantoic fusion in *Mrj* mutants is due to a defect in $\alpha 4$ integrin function. This issue would be resolved by directly assessing cell surface expression and VCAM1 binding activity.

VCAM1 and $\alpha 4$ integrin are the only molecules which are implicated in chorioallantoic fusion to date (Cross et al., 1994; Downs, 1998; Rinkenberger et al., 1997). Notably, mouse mutants for these factors show a variably penetrant effect on chorioallantoic fusion (Gurtner et al., 1995; Kwee et al., 1995; Yang et al., 1995), in contrast to the *Mrj* mutant phenotype which was fully penetrant. This implies that *Mrj* affects another pathway. In exploring trophoblast subtype-specific gene expression we

indeed detected changes in two chorion-specific transcription factor genes; downregulation of *Err2* and an apparent absence of *Gcm1* expression. *Err2*-deficient mouse mutants lack chorionic structures, thus implicating *Err2* in chorion cell proliferation (Luo et al., 1997). The function of *Gcm1* in the chorion is unknown. Whether the reduction of *Err2* and *Gcm1* expression in *Mrj* mutants is a primary part of the phenotype or is secondary to other events is not clear. The effect on *Gcm1* is likely a more direct consequence of *Mrj*-deficiency because we failed to observe even its early expression prior to E8.5.


## DnaJ-related proteins are activators of HSP70 chaperones

The only recognizable motif in the MRJ sequence was the J domain at the N-terminus of the protein. Based on the conserved function of J domains, it is likely that MRJ functions as a co-chaperone with an HSP70. It is through the J domain that DnaJ-like proteins interact with HSP70s and stimulate their ATPase activity (Burston and Clarke, 1995; Caplan et al., 1993). ATP hydrolysis allows conformational changes in the HSP70 necessary for the binding and release of unfolded proteins (Hartl, 1996). This activity has been well characterized for *E.coli* DnaJ (Polissi et al., 1995) and several yeast homologues (Cyr et al., 1994). Distal to the J domain of MRJ, there are 6 Gly residues which are conserved with *E.coli* DnaJ; in other DnaJ-related proteins, a Gly/Phe-rich region occurs at the same position. This region may form a flexible linker between the J domain and the rest of the protein (Pellecchia et al., 1996; Qian et al., 1996).

The remainder of MRJ protein differs from *E.coli* DnaJ but shares regions of similarity with three mammalian DnaJ-like proteins, MSJ1, HSJ1 and HSP40. The significance of these regions of similarity is unknown. However, it is noteworthy that BLAST searches with these motifs revealed that they were present only in these DnaJ-related proteins. MSJ1 (Berruti et al., 1998) bears the greatest sequence similarity to MRJ; both are 242 amino acids in length and overall MSJ1 is 77% identical and 86%

similar to MRJ. Unlike *Mrj*, *Msj1* expression appears to be specific to post-meiotic male germ cells in the testis, a pattern similar to that of *Hsc70t*, a testis-specific member of the HSP70 family (Berruti et al., 1998). *Hsj1* is predominantly expressed in neurons (Cheetham et al., 1992), whereas HSP40 is widely expressed (Ohtsuka, 1993; Raabe and Manley, 1991). The precise functions of these MRJ-related proteins have not been identified.

Beyond these proteins, EST database searches suggested that 40 or more different genes encode J domain proteins in mice and humans. Given the highly specific nature of *Mrj* function, it will be interesting to determine if all each of these genes have a unique cellular and molecular function. In the budding yeast *S. cerevisiae*, mutations in the eight DnaJ-like genes produce distinct phenotypes (Cyr et al., 1994). Chaperone function has been implicated in normal protein folding and re-folding after cell stress (e.g., the heat shock response), intracellular protein trafficking and protein-protein interactions (Hartl, 1996). The specificity in J protein function in yeast is accounted for in part by distinct cellular localization of the proteins. However, it has also been suggested that individual J domains have distinctive activities since swapping J domains, between proteins which are normally localized to different organelles, renders them non-functional (Schlenstedt et al., 1995). The J domain protein specificity is thought to reflect a restricted interaction with different HSP70s, of which there are 14 in yeast (James et al., 1997), as well as different substrate binding abilities. In humans, 11 *Hsp70*-related genes have been identified so far (Tavaria et al., 1996) compared to over 40 different J domain proteins. Identification of the HSP70 associated with MRJ and its substrates will help define MRJ's role(s) in development.

**Role of chaperones during development**

Chaperone activity has been implicated in a variety of cell functions and maintenance of cell viability. Nonetheless, there are only a few examples in higher eukaryotes of

chaperones whose essential functions have been identified by loss-of-function gene mutations. From those that have been studied, however, it is clear that individual chaperones can have very specific functions during development. In *Drosophila*, the *lethal(2) tumorous imaginal discs (l(2)tid)* gene encodes a J domain protein which is involved in imaginal disc cell differentiation (Kurzik-Dumke et al., 1995). The most severe *l(2)tid* mutations lead to overproliferation of undifferentiated imaginal disc cells. In mice, *Hsp70-2*-deficiency results in arrest of spermatogenesis (Zhu et al., 1997). The defect is associated with failure of cdc2 to associate with cyclin B1 during meiosis and thereby form the active mitotic cyclin/cdk complex (Zhu et al., 1997). Notably, the mice are otherwise normal indicating either that chaperone activity for cyclin B1/cdc2 complex formation is not required during mitosis or oogenesis, or that other HSP70s function in these processes. The periodic expression of β-galactosidase along the seminiferous tubule and its localization to germ cells indicates that *Mrj* expression is regulated during spermatogenesis. This pattern of expression potentially overlaps with that of Hsp70-2, suggesting the possibility that MRJ could interact with Hsp70-2 in the testis.

Our analysis of *Mrj* indicates that it is fairly widely expressed beginning early in development. Despite this, its function during early gestation is apparently only required in trophoblast cells of the chorion. Importantly, *Mrj*-deficiency was not associated with loss of chorion cell viability but rather with specific defects in chorion cell function including failure to express cell type-specific genes and permit attachment of the allantois. The specificity of the phenotype re-affirms the idea that DnaJ-related proteins and their associated co-chaperone activities play precise roles in mammalian development.

CHAPTER THREE

CONCLUSIONS AND FUTURE WORK

## Summary

The *Mrj* gene, which was identified using gene trapping in embryonic stem cells, encodes a protein that is related to the DnaJ chaperone of *E. coli*. The N-terminus of the MRJ protein contains a J domain, so named because of its similarity to the N-terminus of DnaJ. This domain has been identified in a wide range of proteins found in yeast, *C. elegans*, and *Drosophila* and at least 40 distinct proteins in mice. Studies done in yeast and *E. coli* indicate that J domain proteins function as co-chaperones for members of the Hsp70 family. It has been suggested that the large number of DnaJ-like proteins in mammals may confer specificity of interaction between a few widely expressed Hsp70s and their substrates (Silver and Way, 1993).

*Mrj* was found to be expressed throughout development in different cell types; notably, at high levels in trophoblast cells of the placenta, neurons of the eye, brain and spinal cord, and germ cells of the testis. *Mrj* expression in the trophoblast lineage included trophoblast giant cells and trophoblast cells of the chorion and their derivatives in the labyrinthine layer of the placenta. *Mrj* gene expression could not be detected in the tissues of mice which carried two copies of the "gene trap allele". Therefore, the gene trap insertion appeared to have created a null allele of the *Mrj* gene. Homozygous mutant embryos did not develop past E9.25 and displayed a failure in attachment of the allantois to the chorion, an event which normally occurs at E8.25 and is essential for development of the labyrinth. Both the chorion and the allantois of *Mrj* homozygous mutants initially appeared to form normally and, at E8.5, expressed the cell adhesion molecules, α4 integrin and VCAM1, respectively. However, expression of the chorion-specific transcription factors *Err2* and *Gcm1* was significantly reduced. These defects of gene expression and receptivity of the chorionic trophoblast cells in the *Mrj* mutants demonstrate a specific and unexpected role for chaperone function in placentation.

**Future Direction 1: Functions of *Mrj* outside of Chorioallantoic Fusion**

Careful analysis of *Mrj*-deficient embryos revealed that the only recognizable defect was failure of the allantois to attach to the chorion. This defect in placentation precluded further development and the embryos arrested at around E9.25. Growth arrest at E9.25 is consistent with other mutations resulting in failure of chorioallantoic attachment such as deficiencies in α4 integrin and VCAM1 (Gurtner et al., 1995; Kwee et al., 1995; Yang et al., 1995). Our observations suggest that up to E9.25, the *Mrj* gene product is only required for chorioallantoic attachment at E8.25 despite the fact that the gene is expressed outside the developing placenta and from an even earlier time in development. Because the embryos died, we could not assess the function of *Mrj* in other tissues or later in the placenta.

Chimeric embryos generated from the aggregation of wild type and mutant embryos and/or cells can be used to assess the developmental potential of cells with a gene deficiency. The presence of sufficient wild type cells allows rescue of the lethal effects of the gene deficiency in a particular tissue (McLaren, 1976). For genes such as *Mrj*, whose deficiency affects extraembryonic cell lineages, we can take advantage of the discovery that when aggregated with diploid embryos the developmental potential of tetraploid embryos is limited to extraembryonic cell lineages and that they rarely contribute to the embryo proper (Nagy and Rossant, 1993). Furthermore, in diploid-tetraploid aggregation chimeras, the trophoblast lineage is primarily derived from the tetraploid cells. Placental defects of *Mash2* and *Hand1* null embryos have been successfully rescued by the extraembryonic contribution of wild type tetraploid cells (Guillemot et al., 1994; Riley et al., 1998). Tetraploid embryos are prepared by the electrofusion of two-cell stage embryos which are then cultured to the 4-cell stage before they are aggregated.

To discover roles for *Mrj* outside of the chorion, embryos produced from heterozygous crosses should be aggregated with wild type tetraploid embryos. Aggregated blastocysts should be transferred to pseudopregnant females. At first, progeny should be genotyped at the time of weaning and surviving homozygous mice should be examined for a mutant phenotype. If homozygous mice are not present among the surviving progeny, this would suggest that they are dying in utero. The tetraploid aggregations should be repeated and embryos should be dissected at various gestational ages after E9.25 and genotyped. *Mrj* mutant embryos which are successfully rescued by tetraploid-derived trophoblast cells based on normal chorioallantoic fusion and survival past E9.25 can be examined for a mutant phenotype. Based on our work to date, we predict that the contribution of wild type trophoblast cells to the chorions of *Mrj* mutant embryos will enable chorioallantoic attachment to occur. Consequently, *Mrj* mutant embryos may have a mutant phenotype at some point in the development after chorioallantoic attachment. Because *Mrj* is widely expressed, we cannot predict what phenotype, if any, will be observed. This phenotype could be related to any of the many other sites of *Mrj* expression during development. *Mrj* is highly expressed in the testis in utero and in the adult as well as in the nervous system and the thymus. Embryos with defects in these tissues would likely survive to term.

## Future Direction 2: Identification of Cell-Autonomous Roles for MRJ

If a gene is required the cell where it is expressed for the normal function of that cell, the gene functions cell autonomously. If the gene is required for the function or development of a cell where it is not expressed, the gene product functions non-cell autonomously. Genes that function non-cell autonomously could encode proteins that are exported from the cell in which they are expressed such as growth factors, cytokines, hormones, extracellular matrix components and proteins in the biosynthetic pathways of

these. Secreted DnaJ-like proteins have not been described previously. However, they have been implicated in general protein export pathways such as ER translocation (Zimmermann, 1998).

To test the cell autonomy of *Mrj* function in the chorion, chorions composed of wild type and mutant cells should be examined for patterns of allantoic attachment. Embryos produced from heterozygous crosses aggregated with wild type tetraploid embryos as described above, could be dissected between E8.5-E8.75 which is after normal chorioallantoic attachment and before extensive labyrinth development. For this experiment, an equal contribution of wild type and mutant cells to the chorion is desirable. This contribution can be rapidly assessed under UV light if the tetraploid embryos contain a transgene allowing for the constitutive expression of green fluorescent protein (GFP). Placentas and allantoises can be sectionned together and the remainder of the embryo and yolk sac can be used for genotyping to identify chimeric placentas derived from *Mrj* mutant cells. Sections should be stained for β-galactosidase activity to identify the *Mrj* mutant cells in the chorion. In *Mrj* mutant diploid - wild type tetraploid chimeric placentas, if the allantois is only attached to the white (β-galactosidase negative) wild type cells, this would suggest a requirement for *Mrj* in the chorionic trophoblast cell for its function and, therefore, a cell autonomous role. If the allantois appears to attach to both white and blue (wild type and mutant) cells, this would suggest a non-cell autonomous role for *Mrj*. In this case, the presence of *Mrj* in some cells can effect either neighbouring chorion cells or the allantois. If the chorions of *Mrj* mutant embryos are consistently derived only from white wild-type cells, this would suggest an intrinsic requirement for *Mrj* for the development of chorionic trophoblast cells.

The major drawback to the above described experiment is that tetraploid embryos tend to overwhelm the trophoblast component of the placenta (J. Rossant, unpublished observations). An alternative approach is to perform the same analysis of chorioallantoic attachment in the placentas of embryos produced from crosses between heterozygotes

aggregated with wild type diploid embryos. This would allow *Mrj* mutant cells to contribute to any part of the embryo, including the placenta, without bias. Again, GFP expression in the diploid embryos would assist in the rapid assessment of wild type cell contribution to the chorion. However, for the purpose of genotyping the conceptuses, the two *Mrj* null alleles must differ in a feature that is detectable by Southern blot analysis so that homozygous contribution can be distinguished from heterozygous contribution in the presence of wild type alleles of *Mrj*. Two distinct null alleles of *Mrj* can be obtained either by discovery of a mouse strain-dependent restriction enzyme polymorphism in the *Mrj* locus or by targetting a new null allele of *Mrj*.

Another technique which may enable the analysis of mosaic placentas for the discernment of a cell-autonomous role for *Mrj* in the placenta is the injection of wild type trophoblast stem (TS) cells into blastocysts produced from crosses between heterozygotes. TS cells can be maintained as stem cells on EMFI feeder cells in media supplemented with FGF4 and heparin or allowed differentiate into trophoblast giant cells in culture as indicated by the expression appropriate marker genes (S. Tanaka, T. Kunath, A. Hadjantonakis, A. Nagy and J. Rossant, in preparation). Importantly, TS cells can be injected into blastocysts where they will contribute only to the trophoblast population (S. Tanaka, T. Kunath, A. Hadjantonakis, A. Nagy and J. Rossant, in preparation). The contribution of TS cells to the placenta ranges from 30 to 60 per cent in the resulting chimeras (T. Kunath, personal communication). Use of TS cells which express GFP would enable the assessment of the contribution of TS cells to the trophoblast population of the chimeras under UV light following dissection. Patterns of chorioallantoic attachment can be examined using histochemistry as described above.

**Future Direction 3: *Mrj* Function in Chorion Receptivity**

Since *Mrj* is expressed in the chorion and not in the allantois, the failure of chorioallantoic attachment in *Mrj* mutants is likely due to a lack of receptivity of the chorion to the allantois. Other gene deficiencies which produce a lack of chorionic receptivity are of interest. To date, the only other mutations which produce this phenotype for genes expressed in the chorion involve genes encoding FGFR2 and α4 integrin (Xu et al., 1998; Yang et al., 1993). To determine if MRJ regulates α4 integrin, its expression was detected by immunostaining. α4 integrin was found to be normally expressed in the chorions of *Mrj* mutant conceptuses. This result indicates that α4 integrin is translated in chorionic trophoblast cells of *Mrj* mutants. It does not prove that it is expressed on the cell surface, however, because both sectioning and acetone treatment in standard immunohistochemical procedures are disruptive to cell membranes. Therefore, it is possible that intracellular protein is also detected. Even if an integrin is at the cell surface, it may not be functional due to a requirement for activation by "inside-out" signaling (Ginsberg et al., 1992). For these reasons, it is important to determine if α4 integrin is present on the surface of the chorion and if it is capable of binding VCAM1 at the time when the chorion should be receptive to allantoic attachment. To test if α4 integrin is present on the cell surface, whole mount immunocytochemistry without membrane permeablization should be performed on the chorion cell surface of intact conceptuses at around E8.5 using the α4 integrin antibody. To study cellular VCAM1 binding capacity, a soluble recombinant VCAM-IgG fusion protein generated at Biogen Inc (Lobb et al., 1995) can be used. By using this reagent and whole mount immunocytochemical procedures, the VCAM1 binding capacity of surface α4 integrin can be detected. A failure to detect α4 integrin on the surface of the *Mrj* mutant chorions would suggest that MRJ protein is involved in the cell surface presentation of α4 integrin. Surface detection of α4 integrin combined with a failure to detect VCAM1 binding

activity on the surface of *Mrj* mutant chorions would suggest that α4 integrin is expressed on the chorion cell surface, but MRJ is required in some way for α4 integrin activation, possibly by the "inside-out" signaling mechanism. Detection of soluble VCAM-IgG on the surface of *Mrj* mutant chorions would suggest that α4 integrin is present and functional in the absence of MRJ protein function. This would suggest the existence of a mechanism of chorioallantoic attachment which is independent of the α4 integrin-VCAM1 interaction.

**Future Direction 4: Identification of MRJ-Interacting Proteins**

There is considerable information concerning the basis of J domain protein specificity which suggests two principles. First, DnaJ-like proteins always function in concert with a specific member of the Hsp70 family of proteins. The interaction between DnaJ-like proteins and members of the Hsp70 family of proteins has been shown using *E. coli*, yeast and human proteins to occur between the J domain of the DnaJ-like proteins and the ATPase domain of Hsp70s. Second, each DnaJ homologue in yeast has a specific intracellular localization that restricts its substrates.

Given the high conservation of amino acid sequence for in all J domains, these domains are predicted to have a similar structure. Immediately distal to the J domain in most homologues (exceptions are those which are membrane-embedded) is a glycine-rich or G/F-rich region thought to form a linker or a flexible hinge between the independently folded J- and C- terminal domains (Pellecchia et al., 1996). In DnaJ, the J domain interacts with DnaK while the C terminal zinc finger-like domain interacts with nascent proteins as they emerge from the ribosome (Gaitanaris et al., 1994; Hendrick et al., 1993). DnaJ-like proteins show more sequence divergence in their C-terminal domains. Therefore, specificity of protein-protein interaction involving MRJ may occur through the C-terminal domain. We hypothesize that N- and C- terminal domains of MRJ are

interacting with different protein subgroups; the N-terminal J domain is interacting with Hsp70-like proteins and the C-terminal is interacting with unknown substrates. Therefore, N- and C-terminal truncated proteins, as well as full length MRJ, should be used as baits for studying biochemical interactions. Construction of N- and C-terminal truncations of MRJ can take advantage of the *Eco*RI site located directly C-terminal to the J domain and G-rich domain. Full length *Mrj* coding sequence and truncations will be subcloned into His6-tagged expression vectors, expressed in *E. coli*, and proteins will be purified by using Ni-affinity chromatography.

For biochemical studies, a polyclonal antibody should be raised against the C-terminal region of MRJ in rabbits and affinity purified using the bacterially expressed C-terminal region of MRJ immobilized on a Ni-sepharose column.

## Intracellular Localization of the MRJ Protein

Biochemical experiments discussed in this section require a cell line that expresses *Mrj* and its interacting protein factors for preparing cell extracts. Since the function of MRJ in trophoblast cells is of particular interest, all available trophoblast cell lines will be tested for *Mrj* expression. Rcho-1 (rat choriocarcinoma) cells differentiate into trophoblast giant cells in culture as indicated by morphology and the expression of appropriate marker genes (Faria and Soares, 1991; Hamlin et al., 1994). Therefore, these cells are likely to be a useful resource, though *Mrj* expression has not yet been confirmed. Expression of the *Mrj* transcript in Rcho-1 cells as well as other trophoblast cell lines available (JAR, JEG-3) will be detected by Northern blot analysis. The intracellular localization of MRJ protein will be determined in a trophoblast cell line where it is expressed using indirect immunofluorescence with the MRJ antibody.

## Identification of a MRJ-interacting Hsp70

To identify an Hsp70 that interacts with MRJ, the placental expression patterns of Hsp70s should first be determined and compared with *Mrj* expression. To date, three Hsp70 genes with placental expression have been identified. The first is *NST-1* which was identified as an EST in an ectoplacental cone library (P. Hunter and J. Cross, unpublished data). The second is heat inducible *HSP70-1* (*HSP68*) which is specifically upregulated in the placenta and yolk sac of E10.5 embryos (Kothary et al., 1987). The third is mouse *HSC70*; the widespread expression of this gene in all tissues has been shown to include the placenta (Kothary et al., 1987). Plasmids containing murine *NST-1*, *HSP70-1* and *HSC70* cDNA should be obtained and the placental expression patterns of these genes should be determined by in situ hybridization on histological sections of E7-13 conceptuses using standard procedures. The cross-hybridization of Hsp70 probes on northern blots observed in previous experiments underscores the importance of obtaining specific probes to detect Hsp70 transcripts (Kothary et al., 1987). Use of 3' UTR sequences for the generation of specific riboprobes will likely circumvent this problem. Analysis of serial sections with a *Mrj* probe will indicate if expression of *Mrj* and any of the *Hsp70*-like gens overlap.

Specific Hsp70s which demonstrate overlapping expression patterns with *Mrj* in the trophoblast lineage can then be tested their ability to interact with MRJ protein in vitro. Interactions will be tested using affinity chromatography. Candidate Hsp70 cDNAs will be cloned into transcription vectors and corresponding proteins will be produced by in vitro transcription/translation. Affinity purified recombinant full length MRJ, as well as N- and C-terminal truncated proteins used as controls, bound to a Ni-sepharose column are mixed with individual Hsp70 proteins. After washing, bound proteins are eluted and characterized by SDS-PAGE. This method has identified an interaction between Hsc70 and Hsp40 using recombinant His-tagged Hsp40 (Minami et al., 1996). However, the

interaction was only observed when an unfolded luciferase substrate and ATP were also added. It is possible that an Hsp70 and DnaJ-like proteins require a substrate to promote their interaction.

Co-immunoprecipitation experiments can be carried out to complement the findings of affinity chromatography or as an alternative method of identifying the Hsp70 which interacts with MRJ. Immunoprecipitation experiments could be carried out using lysates from a cell line that expresses MRJ using the MRJ antibody and a chemical crosslinker as described by others (Yamane et al., 1995). Proteins that coprecipitate with MRJ can be characterized using SDS-PAGE and western blotting. Initially, an antiserum available from Sigma, which crossreacts with many Hsp70s, can be used to detect Hsp70s. Specific Hsp70s may be recognized by their precise mass; for example HSP70-1 runs at 68 kD and HSC70 runs at 74 kD. To confirm the identity of the Hsp70 which co-precipitates with MRJ, a specific antibody should be obtained if available.

**Identification of Hsp70 - MRJ Substrates**

Proteins other than Hsp70s that co-immunoprecipitate with MRJ may be specific substrates or co-chaperones of the putative Hsp70-MRJ chaperone activity. Without any knowledge of such interacting proteins, the only means for their identification is microsequencing using mass spectrometry. Recent advances in protein sequencing technology have led to the development of Q-TOF which is a mass spectrometer capable of sequencing femtomolar quantities (Morris et al., 1997). To sequence proteins appearing on silver or Coomassie stained SDS-PAGE gels that co-precipitate with MRJ the unknown band should first be excised from the gel followed by extraction using a protocol that does not use paraformaldehyde which permanently modifies the protein (Shevchenko et al., 1996). The sample can be digested with trypsin while it is still in the

gel or following extraction. Extracted peptides would be partially sequenced by Q-TOF and then BLAST searches could be used to identify the full length protein.

The yeast two hybrid system has been widely used as a screen to identify proteins that interact with a protein of interest. According to this system, MRJ would be expressed from a plasmid in yeast as a fusion protein containing either the LexA or Gal4 DNA binding domains (BD). A mouse embryo cDNA library cloned into a yeast plasmid designed to express the contained cDNA as a fusion protein with the VP16 transcriptional activation domain (AD) would be obtained (Hollenberg et al., 1995). The host yeast strain would contain a selectable marker and a reporter gene both located downstream from several copies of either the LexA or Gal4 enhancer elements. To test the MRJ-BD fusion proteins for endogenous transcriptional activation capability, the BD plasmid should be transfected into the host strain alone and assayed for reporter gene activity. Then the cDNA-AD plasmids would be transfected and maintained in the host strain. Clones are then subjected to selection for marker/reporter expression. In theory, for individual clones to survive selection, the cDNA on the AD plasmid must encode a protein that interacts with MRJ and it must be expressed as a fusion protein with the activation domain. cDNA fragments of plasmids encoding putative MRJ interacting proteins will be sequenced and the BLAST program would be used to search for homologous proteins in the NCBI database. Follow-up tests should be carried out to eliminate the false positives that frequently appear when using the yeast two hybrid system. For example, proteins that activate transcription without interacting with MRJ can be eliminated by transforming an untagged version of MRJ into the positive yeast clone, immunprecipitating the putative interacting protein with the MRJ antibody and detecting it by western blot with an antibody against the AD part of the fusion protein which can be obtained from Sigma.

The yeast two hybrid method was used to identify Hip, an Hsc70-interacting protein in which the ATPase domain of Hsc70 was used as bait (Hohfeld et al., 1995).

Since this domain is bound by Hsp40 and perhaps other DnaJ-like proteins, Hsp40 might have been expected to be identified in this screen. However, there was a notable absence of J domain-containing proteins identified in this screen; Hip was the only interacting protein identified. Because *Mrj* expression overlaps with HSP70-2 in the testis, a collaborator tested MRJ for its ability to interact with HSP70-2 using the yeast two hybrid system. They were unable to detect an interaction. It is possible that the hypothesized transient interaction between Hsp70s and J domain containing proteins is not amenable to the assay of the yeast two hybrid system. Nevertheless, if an Hsp70 which interacts with MRJ has been identified at the time that this screen is undertaken, its cDNA should be subcloned in frame into the AD plasmid and tested for its ability to interact with MRJ under yeast two hybrid conditions. However, the main reason to undertake a yeast two hybrid screen with MRJ is for the identification of interacting proteins other than Hsp70s and for this purpose, it is a potentially useful tool.

## Conclusions

Future work should proceed with the general hypothesis that MRJ confers specificity of Hsp70 chaperone activity to a subset of cellular proteins, some of those being specifically required for chorioallantoic attachment. The cellular function of MRJ can be clarified by (1) identifying other tissues that require its function in development, (2) determining if its function is cell autonomous, (3) testing for participation in the $\alpha 4$ integrin - VCAM1 interaction and (4) determining the intracellular localization. These results may provide clues to the identity of potential Hsp70 partners and substrates of MRJ. In the meantime, the identity of substrates and the Hsp70 interacting partner can be sought by assaying candidates and screening libraries for biochemical interaction. By identifying the molecular processes in which MRJ participates, this should yield insights into chorioallantoic attachment, an event that is not well understood. This may have

important implications for the general interaction between mesoderm and trophoblast in other eutherian mammals. More generally, as the *Mrj* mutants are the first example of the effects of a J domain protein defect in higher eukaryotes, more detailed study will provide interesting insights into the specific cellular functions of these co-chaperones.

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