Mid-gestational lethality in mice lacking keratin 8

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Keratin 8 (mK8) and its partner keratin 18 (mK18) are the first intermediate filament proteins expressed during mouse embryogenesis. They are found in most extraembryonic and embryonic simple epithelia, including trophoctoderm, visceral yolk sac, gastrointestinal tract, lungs, mammary glands, and uterus. We report that a targeted null mutation in the *mK8* gene causes mid-gestational lethality. Mutant embryos are growth retarded and suffer from internal bleeding, with an abnormal accumulation of erythrocytes in fetal livers. The *mK8*⁻ phenotype has 94% penetrance, with a few mice surviving into adulthood. We suggest that mK8/mK18 filaments are important for the integrity of the fetal liver, like specialized human epidermal keratins for the integrity of the epidermis. This phenotype in mice differs from the reported function of simple epithelium keratins in *Xenopus* at the gastrulation stage. In mice, mK8 fulfills a vital function at 12 days postcoitum.

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The two types of keratins constitute the largest family of intermediate filament (IF) proteins. The 22 identified members are obligate heteropolymers requiring one type I and one type II keratin for the formation of extended keratin filaments. They are expressed as sets of one, two, or three pairs in diverse epithelia (for review, see Steinert and Roop 1988). Despite many studies on the structure of keratin filaments and their spatiotemporal regulation in normal and tumorigenic tissues, little is known about keratin function(s). Disruption of intermediate filaments in cultured cells does not interfere with functions that are associated with cytoskeletal proteins, such as cell shape, cell migration, or cell adhesion (Klymkowsky et al. 1983; Boller et al. 1987; Baribault and Oshima 1991). Similarly, the disruption of keratin filaments does not prevent the development of mouse embryos to the blastocyst stage (Emerson 1988). These intriguing but negative results challenged the assumption that keratin filaments are structural proteins and led to the suggestion that keratin filament function might be revealed within the context of a whole organism. Transgenic mice expressing a dominant-negative form of K14 suffer from skin abnormalities resembling a human genetic disease (Coulombe et al. 1991a; Vassar et al. 1991). These findings have provided strong evidence that epidermal keratins are required for the structural integrity of the skin. Moreover, point mutations in human epidermal keratins, such as the K1/K10 and K5/K14 pairs, are associated with inherited genetic skin diseases, such as epider-

molysis bullosa simplex (Bonifas et al. 1991; Coulombe et al. 1991b; Lane et al. 1992) and epidermolysis hyperkeratosis (Cheng et al. 1992; Chipev et al. 1992; Rothgangel et al. 1992). In *Xenopus* embryos, keratin depletion by either antisense oligonucleotide or antibody microinjection prevents gastrulation (Klymkowsky et al. 1992; Torpey et al. 1992).

Keratin 8 (mK8) and its partner keratin 18 (mK18) are the first IF proteins to be expressed during mouse embryogenesis (Jackson et al. 1980; Kemler et al. 1981; Oshima et al. 1983). They are expressed in several extraembryonic tissues, including the trophoctoderm, the endoderm, the visceral yolk sac, and the placenta. In embryonic tissues, they are found in the gastrointestinal tract, the lungs, the uterus, and most other simple epithelia (Moll et al. 1982). To study mK8/mK18 filament function, we have generated cells and mice deficient in *mK8* by gene targeting in embryonic stem (ES) cells. This mutation prevents keratin filament formation in several epithelia, because *mK8* is the only type II keratin to be expressed in the trophoctoderm, endoderm, hepatocytes, and most intestine epithelial cells and because type I mK18 and mK19 are unable to form filament without their *mK8* type II partner.

Previously, we reported that the targeted inactivation of both *mK8* alleles in ES cells did not prevent the formation of a polarized and functional extraembryonic endoderm epithelium in an in vitro embryoid body formation assay (Baribault and Oshima 1991). We have now determined the effect of a genetic *mK8* deficiency on the development of the mouse. Blastocysts lacking mK8 im-

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plant normally and develop beyond gastrulation without apparent defect. Yet, *mK8* deficiency causes embryonic lethality between 12 and 13 days of embryogenesis, at a time where fetal hematopoiesis migrates from the yolk sac to the liver. This mutation is recessive and has 94% penetrance. The growth of the mutant embryos is retarded, and internal bleeding is often observed. These results demonstrate that simple epithelial keratins are important for the continued development of the mid-gestational mouse embryo.

Results

Targeted *mK8* mutation

We have introduced a targeted *mK8* mutation into ES cells as reported previously (Baribault and Oshima 1991). The targeting vector contains two arms of nonisogenic *mK8* genomic DNA of 1.8 and 1.2 kb, separated by a *neo^r* gene, pMC1neopolA (Fig. 1). The *neo^r* gene replaced most of the first exon, including the ATG translation initiation codon, to prevent protein synthesis should there be residual transcription at the targeted locus. The herpes simplex virus—thymidine kinase (HSV-tk) gene was added 3' of the targeting vector to make use of the positive/negative selection and to enrich for targeting events (Mansour et al. 1988). Linearized DNA was electroporated in D3-ES cells. A targeting frequency of 1/30 screened colonies was obtained. Homologous recombination events were confirmed by Southern blot analysis (Fig. 2A). From the four targeted ES clones analyzed, two

were tested *in vivo*. One clone contributed to the germ line of chimeric mice after blastocyst injection, and the targeted mutation was transmitted to the progeny (Fig. 2B,C).

After homologous recombination, in all clones analyzed from the transfection of E14TG2a and D3-ES cells, a single copy of the targeting vector was found at the targeted locus, indicating that the pMC1neopolA regulatory elements are sufficient to drive the *neo^r* expression at this integration site.

mK8 deficiency causes embryonic lethality

The mouse colony carrying an *mK8⁻* targeted mutation was expanded by breeding heterozygous progeny to wild-type C57Bl/6 mice. *mK8⁻* heterozygous male and female mice were bred, and the percentages of homozygous progeny were determined at different stages of embryogenesis and after birth in newborn and adult mice (Figs. 2D and 3). The genotype of embryos up to 8.5 days was determined by *mK8* immunofluorescence staining. For later stages, Southern blot analysis was performed on DNA isolated from the visceral yolk sac or the tip of the tail. At most stages, nearly 100 progeny were analyzed to provide a statistical standard deviation of no more than 2%. Homozygous embryos were found at a frequency of ~25% up to day 9.5 days postcoitum (p.c.), well beyond the gastrulation stage at 8 days p.c. A dramatic decrease in the recovery of homozygous embryos, between 12 and 13 days of embryogenesis, shows that *mK8* deficiency

Figure 1. *mK8* targeting vector. The genomic structure of the *mK8* gene is represented at top (Tamia et al. 1991). The *mK8* targeting vector contains two arms of homology to the *mK8* gene. The *neo^r* gene replaces most of the first exon, including the ATG translation initiation codon. HSV-tk was added 3' of the targeting vector followed by the Bluescript plasmid DNA. The targeting events were identified by PCR screening of the G418/GanC-resistant clones using two oligonucleotides represented by rectangular boxes. Clones with the expected PCR product (represented by a double strand) were expanded and analyzed by Southern blot analysis using the Endo A $\alpha 2$ probe homologous to the exonic *mK8* sequences and the *XhoI*–*XbaI* fragment located 5' of the *mK8* gene (5' probe). The expected length for the wild-type and targeted *XbaI*, *HindIII*–*Asp718*, and *XhoI*–*HindIII* fragments are shown. We found a polymorphism for the *XbaI* restriction site between the 129 and C57Bl/6 mouse strains, respectively, leading to an 8.3- and a 5.5-kb restriction fragment, respectively.

Figure 2. Southern blot analysis of *mK8*⁻ targeted ES cells and mice carrying the *mK8*⁻ targeted mutation. Five micrograms of genomic DNA isolated from parental D3-ES cells (A, lanes 2,8), from the PCR-positive targeted clones mK8-KO-1 to mK8-KO-4 (A, lanes 3-6, 9-12) or from a PCR-negative *neo*^r control clone (A, lanes 7,13), were digested with *Hind*III-Asp718 (A, lanes 2-7) or *Xba*I (A, lanes 8-13) and were loaded in each lane. After homologous recombination, the targeted clones had one wild-type (wt) and one targeted (*mK8*⁻) allele, with the expected molecular mass as seen by hybridization of the filter with the EA-2α probe, homologous to the mK8 exonic sequence (A). (psg) Hybridization with the *mK8* pseudogene. Radioactive λ *Hind*III markers were loaded in lanes 1 and 14. The mK8-KO-1 cells contributed to the germ line of a chimera (B). Four micrograms of genomic DNA was digested with *Xba*I and hybridized with the EA-2α probe. (B) (Lane 1) λ *Hind*III markers; (lane 2) DNA isolated from the tip of the tail of an agouti progeny; (lane 3) mK8-KO-1 cells; (lane 4) D3-ES parental cells; (lane 5) liver DNA from a C57Bl/6 mouse. Note the polymorphism between the wild-type allele in 129-derived and C57Bl/6 *Xba*I restriction fragment. The presence of the targeted allele in the *mK8*⁻ breeding colony was further confirmed by *Xba*I (C, lanes 1-5) and *Xho*I-*Hind*III (C, lanes 6-10) digestion and hybridization with the EA-R6 *mK8* cDNA probe (lanes 1-5) and 5' probe (lanes 6-10) respectively, of genomic DNA isolated from heterozygous (lanes 1,4,6,9) or wild-type (lanes 2,3,5,7,8,10) progeny. Mice were routinely screened with Southern blot analysis of *Xba*I-digested mouse tail DNA (D). Hybridization took place with either the EA-R6 cDNA fragment allowed to identify the homozygote (-/-), heterozygous (-/+), or wild-type (+/+) progeny resulting from heterozygous breeding (D). Note the presence of a homozygous mouse in D (-/-).

causes mid-gestational lethality. A small proportion of homozygous embryos (6%) escape the embryonic defect and develop to the adult stage. Thus, the embryonic lethal phenotype has an incomplete penetrance of 94%.

The *mK8* targeted mutation eliminates *mK8* RNA and protein

mRNAs in various tissues of homozygous, heterozygous, and control mice were compared by Northern blot analysis (Fig. 4). The *mK8* targeted mutation abolished the presence of normal *mK8* RNA in all homozygous tissues analyzed. No detectable *mK8* transcript could be observed in liver and kidney (Fig. 4A), or in lung and uterus (data not shown). A higher molecular mass transcript was observed in RNA isolated from intestine. This residual *mK8* RNA most likely represents a fusion transcript with the *neo*^r gene because the same size RNA hybridizes with both the *mK8* and the *neo*^r probe (Fig.

4C). The generation of such a fusion transcript could be explained by an alternative usage of the pMC1neopola and *mK8* polyadenylation signal, the latter representing only ~1% of both forms. We quantitated the amount of *mK8* transcript in wild-type gut and found that it represents as much as 300 pg/mg of total RNA or ~3% of the mRNA (Fig. 4E). Thus, the use of pMC1neopola can lead to residual transcription of the targeted locus when the targeted gene product is abundantly expressed. Still, this fusion transcript is not expected to produce a functional protein, as we have removed most of the first exon including the ATG translation initiation codon.

The RNA for the complementary mK18 subunit is found in the same tissues as mK8, although its abundance is much less in intestine and kidney (Fig. 4B). The levels of *mK18* RNA were not affected by the *mK8* deficiency. A high level of the *neo*^r expression was found in intestine (Fig. 4C). The *neo*^r RNA was also detectable faintly in all other tissues analyzed, including spleen,

Figure 3. Recovery of homozygote ($mK8^{-}/mK8^{-}$) progeny at various stages of embryogenesis and in adult mice. (Left) Heterozygous ($htrz$) male and female mice were bred, and the genotype of the progeny was determined by Southern blot analysis and/or the presence of $mK8$ protein. The genotypes of 3.5, 7.5, and 8.5 day embryos were determined by immunofluorescence staining of the $mK8$ protein. For embryos of later stages, Southern blot analysis was performed on DNA isolated from the visceral yolk sac. The genotype of adult mice was determined by Southern blot analysis of tail DNA. (Right) Progeny of homozygous males bred to heterozygous females were examined. The numbers above the points indicate the number of progeny analyzed at each stage. Note that pups were counted the day after birth. Therefore, the numbers of embryos at day 1.5 and 40 are identical, because death did not occur during that time.

(Fig. 4C) and in primary culture of embryonic fibroblasts (data not shown), even though the $mK8$ locus is not normally active in these cells.

As expected, no $mK8$ protein was detected in homozygous embryonic and adult tissues (Figs. 4F and 5). Immunofluorescence staining of heterozygous and homozygous intestine shows that no $mK8$ filament can be detected in homozygous tissues (Fig. 4F). Furthermore, no $mK8$ staining was detectable in homozygous blastocysts, in all extraembryonic tissues, and in embryonic epithelia from 8.5 and 12.5 day embryos (data not shown), and from 17.5 day embryos (Fig. 5).

$mK8^{-}$ mutant embryos are growth retarded and suffer internal bleeding

Almost all homozygous $mK8^{-}$ embryos die at ~12 days of development. Figure 6 shows representative heterozygous and homozygous embryos around the time of lethality, 12.5 days p.c. (A, B, D–K) and 13.5 days p.c. (Fig. 6C). A comparison of littermates heterozygous (Fig. 6A,D) and homozygous (Fig. 6B,G) for the $mK8$ targeted mutation shows that 12.5 day mutant embryos are smaller. The weights of progeny from two litters of heterozygote matings confirmed visual observations and indicated that homozygous embryos were only 65–70% of the normal weight (data not shown). At 13.5 days p.c., mutant embryos are found necrotic and in the process of being reabsorbed (Fig. 6C, right). By day 15.5, homozygous embryos are usually completely reabsorbed.

At 12 days p.c., major steps of organogenesis have already occurred and several organs where $mK8$ is normally expressed are easily recognizable, notably the liver, the lungs, the stomach, and the gut (Rugh 1990). The pancreas, the spleen, and the thymus are still organ primordia. One well-documented feature at day 12 of embryogenesis is the migration of the hematopoietic system from the visceral yolk sac to the liver (Mucenski et al. 1991).

Despite growth retardation, mutant embryos formed all major organs where $mK8$ would normally be expressed (Fig. 7C,D), including liver, gut, and stomach, as well as extraembryonic placenta and visceral yolk sac. In several mutant embryos, internal bleeding was observed (arrows in Fig. 6; better seen by the red color in Fig. 7B). Histological examination of mutant and control 12.5 day embryos revealed an abnormal accumulation of nucleated embryonic erythrocytes in the liver as well as in the neighboring tissues (Fig. 7). In some areas erythrocytes appeared to cross the liver capsule. Other epithelia such as stomach and gut appeared histologically normal. The same phenotype was observed in a mutant embryo sacrificed at 17.5 days of gestation owing to distress and vaginal bleeding of the heterozygous mother.

These results suggest that the embryonic liver, for structural or functional reasons, is unable to support the active hematopoietic growth taking place *de novo* in the hepatic tissue at this stage, and consequently disrupts, allowing erythrocytes to diffuse in the neighboring tissues. This interpretation is supported further by the ob-

Figure 4. Northern blot and immunofluorescence analysis of *mK8* expression in wild-type, heterozygous, and homozygous adult mice. Twenty micrograms of total RNA isolated from various tissues was loaded in each lane. The same filter was hybridized with the EA-R6 fragment of *mK8* cDNA (A), the full-length *mK18* cDNA (B), and the *XhoI-SalI* *neo* fragment from pMC1neopola (C). The filter was then rehybridized with the ribosomal protein L32 gene (D) as an internal control to standardize the amount of RNA loaded in each lane. (E) Synthetic RNA was made from the EA-R6 fragment of the *mK8* cDNA. This RNA was used as a standard to quantitate the amount of *mK8* transcript in the gut of wild-type, heterozygote, and homozygote mice. Amounts of synthetic RNA as well as gut total RNA are indicated above each lane. Ten micrograms of *mK8*⁻ spleen RNA was added to each sample of the gut RNA serial dilutions, as carrier. Approximately 3% of the mRNA in the gut is *mK8* transcript, assuming that the mRNA levels are 1% of the total RNA. The presence of a higher molecular mass transcript in the gut likely results from a fusion transcript with the *neo*^r gene at the targeted locus, as the *neo*^r probe hybridized with a band of the same molecular mass. (F) *mK8* immunofluorescence staining of heterozygous (left) and homozygous (right) intestine, using the TROMA-1 rat monoclonal antibody specific against *mK8*.

servation that *mK8/mK18* is the only IF normally expressed in the liver; consequently, in the absence of *mK8/mK18* filaments, no other IF could complement a hepatic defect. However, *mK8* is expressed in a variety of epithelia at this stage. Other tissues may contribute to the phenotype, particularly the failure to grow.

mK8⁻ phenotype has an incomplete penetrance

Although most *mK8*⁻ embryos die in utero, 6% of the homozygous progeny (or 1.6% of the total progeny) escape the embryonic defect and develop to the adult stage without apparent abnormality (Fig. 3; Table 1). An equal number of male and female escapers were obtained (data not shown). Still, despite several attempts to breed these

mice, we have not been able to establish a homozygous mouse line. The male escapers are fertile but do not transmit homozygosity when bred to heterozygote females (Fig. 3, right). Of the three homozygous females analyzed, one was cannibalized by its littermates (an unusual occurrence of unknown significance) and one was sacrificed for tissue analysis because of distress and bleeding from the vaginal tract after breeding to a homozygous male. In this animal the decidui were still present, but no embryos were found. Presumably, they were already reabsorbed, because they were all homozygous. The third homozygous female was bred to a wild-type male to determine whether it was fertile and capable of sustaining pregnancy. This homozygous female gave birth to two pups, which were found dead at birth. The death of the embryos was not the result of embryonic deficiencies because embryos had to be heterozygous. However, similar deaths are occasionally seen in normal colony breeding as well and may not be specific to the female escaper progeny. These results show that female escapers are capable of producing competent eggs and of sustaining implantation and placental function. However, the question of whether female escapers are completely reproductively competent and capable of lactation remains open.

Histological analysis of the homozygous adult epithelia, where *mK8* is normally expressed, shows no pathological abnormalities. Sections of liver, small and large intestine, stomach, kidney, bladder, pancreas, lung, uterus, and spleen from wild-type, heterozygous, and homozygous mice were compared, and no differences were observed (data not shown).

Although incomplete penetrance is often observed in human genetic diseases and in mutant mouse lines, the causes of this phenomenon are still obscure. It has been

Figure 5. Immunofluorescence staining of mK8, mK18, and mK19 in heterozygous and homozygous embryos. Frozen sections of whole heterozygous (-/+) or homozygous (-/-) 17.5 day embryos were fixed with methanol and stained with TROMA-1 (mK8), TROMA-2 (mK18), or TROMA-3 (mK19) monoclonal antibodies. (Top) mK8, as well as mK18, is absent from the liver of homozygous embryos, except for the lining of the lobes. These results are consistent with in vitro findings that in the absence of a type II partner, mK18 does not form keratin filaments. (Middle) Lungs from homozygous embryos are deficient in mK8. In the absence of mK8, the distribution of mK18 and mK19 is altered, and stained aggregates are visible. This is compatible with the notion that other type II keratins are expressed in the lungs. (Bottom) Gut from homozygous embryos is mK8 deficient. In the absence of mK8, the distribution of mK18 and mK19 is largely reduced, although normal arrays of mK18 filaments remain in rare cells.

argued that the interaction of a given mutation with the carrier genetic background could be a responsible element (LeMouellie et al. 1992). Alternatively, stochastic or epigenetic factors could be involved. We outbred the mK8 genetic mutation to a FVB/N background. After one cross and subsequent breeding of the heterozygous mice, we observed that 9% adult progeny were homozygous (Table 1). This percentage was significantly different from the 1.6% observed with our previous mouse line. These results show that the penetrance of the *mK8*⁻ phenotype is dependent on the mouse genetic background. If only one gene from the FVB/N genetic background complements the embryonic defect, assuming that the FVB/N mouse strain is homozygous at all loci, we would have expected a frequency of 18.8% escapers. The 9% observed here is significantly lower and therefore would indicate that the interaction of the *mK8* mutation with the FVB/N genetic background involves several genes. The proportion of escapers is expected to increase as we cross the *mK8* mutation into a pure FVB/N genetic background for several generations.

The presence of viable homozygous animals may permit the investigation of *mK8* function past the first stage

of lethality. For example, *mK8* could be involved in mammary gland epithelium morphogenesis taking place during lactation, in liver regeneration, after partial hepatectomy, and in carcinoma progression. The study of these phenomena in *mK8*-deficient mice and wild-type littermates may provide direct insights into these hypotheses.

Functional redundancy with other keratins was not observed

Keratins are obligate heteropolymers. The type I mK18 and mK19 keratins cannot form filaments without a type II partner such as mK8. If another type II keratin family member was induced to compensate for the loss of mK8, type I mK18 and mK19 would be expected to form filaments even without mK8 because the copolymerization of different type I and II members is promiscuous (Hatzfeld and Franke 1985). Staining of homozygous and control heterozygous 17.5 day embryos with mK8-, mK18-, and mK19-specific monoclonal antibodies did not reveal compensatory keratin filament systems. The expected mK18 and mK19 staining pattern was found in

Figure 6. Photograph of homozygous ($mK8^{-}$) and heterozygous ($-/+$) embryos at 12.5 (A,B,D–K) and 13.5 (C) days of development. (A,B,D,G) Littermates. Note the difference in size. (E–K) Homozygous embryos affected at various degrees by the mutation. Note also internal bleeding of embryos in B, E, G, and K (arrows), as well as the retarded appearance of embryo E. (C) 13.5 day heterozygous (left) and homozygous (right) embryos. The mutant (right) is shown attached to the placenta (p). Note that it is completely necrotic.

the liver, lung, and gut from homozygous and heterozygous 17.5 day embryos.

In the absence of mK8, mK18 was not detected in hepatic tissues, except for the liver capsule surrounding the hepatic lobes, presumably because an additional type II keratin, besides mK8, is normally expressed in the liver capsule (Fig. 5). mK18 was detected in the lungs of mK8-deficient embryos (Fig. 5) as aggregates of antigenic material rather than the normal dispersed, filamentous pattern found in control and heterozygous animals. The mK7 in lung tissues (Moll et al. 1982) likely results in coassembly with mK18 when mK8 is absent. The mK18 aggregate pattern observed in $mK8^{-}$ lung may be the result of an imbalance of mK18 and mK7 or may be a characteristic of the uncommon pairing of these keratins. These results confirm that the mK18 antibody can recognize mK18 in a heterotypic complex with type II keratins other than its normal mK8 partner. In mK8-deficient intestine, mK18 is absent from almost all epithelial cells. A few apparently specialized cells contain detectable levels of mK18 and mK19 (Fig. 5). It appears that a minority subpopulation of gut epithelial cells express another type II keratin with which mK18 and mK19 can polymerize. Similar results were obtained when 8.5 and 12.5 day embryos were stained for mK18 and mK19. Both proteins were completely absent from the visceral endoderm and hepatic tissues, confirming

that there was no redundancy with other keratin family members in several embryonic and extraembryonic epithelia. Furthermore, as with extraembryonic endoderm formed in vitro (Baribault and Oshima 1991), the trophoblast, the visceral yolk sac, and the liver are not known to express any other members of the IF superfamily. For example, we and others have confirmed the absence of vimentin filaments in these three examples. The absence of an obvious phenotype before day 12 p.c. shows that IFs are dispensable for the formation of trophoblast and other extraembryonic epithelia. In mice, mK8 fulfills a vital function at 12 days p.c.

Discussion

Simple epithelium keratins have a different embryonic function in mammals and amphibians

These findings constitute the first description of a phenotype associated with a simple epithelium keratin mutation in mammals. In *Xenopus*, depletion of keratins by antibody or antisense oligonucleotide microinjection leads to a specific block of the amphibian development at the gastrulation stage. Thus, simple epithelial keratins fulfill a different embryonic function in mammals than in amphibians. These results are somewhat surprising when we consider the conservation of keratins dur-

Figure 7. Histological analysis of 12.5 and 17.5 day homozygous embryos. The homozygous (B) and a control heterozygous (A) embryo are littermates. Paraffin sections of heterozygous (C,E,G) and homozygous (D,F,H) embryos were stained with hematoxylin–eosin. (A–F) 12.5 day embryos. Note the abnormally high abundance of fetal erythrocytes in the liver and neighboring tissues of *mK8*[−] embryos. The same phenotype was observed in a mutant embryo (H) sacrificed at 17.5 days of gestation owing to distress and vaginal bleeding of the heterozygous mother. (G) Liver section of a 17.5 day heterozygous littermate. Magnifications: (C,D) 28×; (E–H) 142×. (he) Heart; (li) liver; (e) erythrocytes.

ing evolution (Steinert and Roop 1990). Still, mouse and amphibian gastrulation are dramatically different. Epithelium reorganization and migration is much more important in the latter. Therefore, the cellular mechanisms by which simple epithelium keratins exert their function could be analogous despite the different embryonic stages at which this function would be required for embryonic development.

mK8 deficiency results in embryonic lethality be-

tween 12 and 13 days of development. The abnormal agglomerations of embryonic, nucleated erythrocytes in liver and other tissues, characteristic of mutant embryos, may reflect a function of *mK8* filaments in the structural integrity of fetal liver. Fetal liver deficient in keratin filaments may be unable to accommodate the rapid proliferation of hematopoietic cells taking place at this stage with resulting disruption and bleeding. However, it seems unlikely that a defect in the structural

Table 1. Percentage of homozygous escapers

Genetic background	<i>mK8</i> [−] / <i>mK8</i> [−]		<i>mK8</i> [−] /+		+/+		Total	
	%	(n)	%	(n)	%	(n)	%	(n)
C57Bl/6 (F ₂₋₆)	1.6	(7)	64.7	(286)	33.7	(149)	100	(442)
FVB/N (F ₂)	9.1	(14)	51.3	(79)	39.6	(61)	100	(154)

The *mK8* null mutation was bred for several generations into a C57Bl/6 genetic background. The offspring from heterozygote breeding from the second to the sixth generations were genotyped by Southern blot analysis (F₂₋₆) C57Bl/6. Subsequently, the *mK8*[−] mutation carriers were bred with FVB/N wild-type mice. The resulting heterozygous (C57Bl/6 × FVB/N) mice were bred and the resulting F₂ progeny were genotyped (F₂) FVB/N. The proportion of homozygous escapers from the *mK8* mutation passaged several times into a C57Bl/6 and once into a FVB/N genetic background is significantly different (*a* < 0.001, Fisher exact test with correction for continuity) [Zar 1984]. (+/+) Wild type; (*mK8*[−]/+) heterozygote; (*mK8*[−]/*mK8*[−]) homozygote.

integrity of fetal liver could account for the growth retardation of the mutant embryos apparent at 12 days of development. Additional defects in other epithelia could affect growth factor production or utilization. Alternatively, a deficiency in *mK8* may impair placental function in nutrient transport or growth factor production. The placenta of the mutant embryos is a mosaic of *mK8*[−] maternal tissues and *mK8*[−] embryonic contributions. An interaction between embryonic and maternal-derived components may contribute to the <100% penetrance of the phenotype. Although no morphological defect has been observed in the *mK8*[−] placenta, one direct test of the functional activity of the placental functions will be to transfer *mK8*[−] inner cell masses to wild-type blastocysts to determine whether the resulting *mK8*⁺ placenta can alleviate mid-gestational lethality.

Embryos and embryoid bodies

The absence of a developmental defect associated with trophectoderm and yolk sac formation shows that the in vitro differentiation of *mK8*[−] doubly targeted ES cells to a functional and polarized extraembryonic epithelium (Baribault and Oshima 1991) was relevant to *mK8* function in vivo. The absence of a *mK8*[−] phenotype during embryoid body formation was unexpected, particularly for a cytoskeletal protein. The ectopic vimentin expression in the undifferentiated ES cells or the lack of physical stress in vitro, equivalent to the implantation and expansion occurring in vivo, could have masked the function of *mK8*. However, the present finding that an embryonic defect is observed well beyond the formation of the egg-cylinder stage that embryoid bodies mimic further reinforces the utility of the model system of ES cell differentiation in vitro. The absence of a phenotype at early stages does not necessarily mean that these filaments serve no purpose at other stages. Rather, additional gene products may supplement the function of *mK8* filaments in both the earliest and adult epithelia. This is supported by the presence of homozygous viable animals that escape the embryonic defect, an observation that suggests the presence of genetic suppressors of the *mK8*[−] phenotype. The hypothetical genes that complement the *mK8* deficiency in trophectoderm, yolk sac, and liver are not other keratins or other IFs because no IFs other than K8/K18 or K8/K19 have been identified in these tissues. However, the different cytoskeletal filament systems, actin, microtubules, and IFs, may be functionally integrated and perhaps should not be considered completely separate (for review, see Goldman et al. 1990; Fuchs 1991).

Functional specificity of IF family members

Individual members of the superfamily of IF genes are expressed in specific patterns, sometimes alone or as a subset of IF proteins, during embryogenesis, and in adult tissues. This complex regulation raises the question of whether individual IF family members are functionally specific or interchangeable.

All IF proteins share a common α -helical domain that is required for subunit polymerization into extended filaments. In contrast, the non- α -helical head and tail domains are highly variable in length and biochemical properties among IF members (for review, see Steinert and Roop 1990). Among the keratin family, the end domains of epidermal and simple epithelial keratins have diverged to glycine- and serine-rich domains, respectively. The glycine-rich domain of epidermal keratins leads to a more stable and less flexible keratin consistent with the protective function of the skin. The serine-rich domains of simple epithelium keratins confer a more flexible structure, compatible with the necessity for IF remodeling during migratory processes and tissue reorganization. Recent studies have clearly shown the structural function of the skin keratins. Still, one must beware of extending this conclusion to all IF family members. There have been reported examples of distinct functions associated with different members of a multi-gene family. For example, the lens δ -crystallin protein clearly has a structural function. Yet, it has been recruited during evolution from similar proteins with enzymatic activities and is closely related to enolase, τ -crystallin and other taxon-specific crystallins (Wistow and Piatigorsky 1987).

The *mK8* null-genetic background now permits individual IF proteins and their terminal domains to be tested for their functional specificity. The closest relative of K8 is the K7 type II keratin. The two proteins share 90% identity in their rod domains but have less homology in their head and tail domains (20–30%). We have already shown that a 10-kb K18 genomic fragment is sufficient to confer complete tissue-specific and copy-number-dependent expression in transgenic mice (Abe and Oshima 1990; Neznanov et al. 1993). Because *mK8* and *mK18* have a near identical expression pattern, the use of this vector to drive K7, various recombinant hybrids of K7 and K8, and other IF proteins such as vimentin may permit a test of the functional specificity of these proteins.

Null and dominant-negative mutation

All reported examples of inherited diseases associated with keratin genetic alterations are dominant, even though some recessive forms of epidermolysis exist and could well be the result of keratin null mutations. These genetic alterations are point mutations in the rod domain that act as dominant-negative mutations. Still, the question of whether dominant-negative and null mutations produce the same phenotype remains open. A wide variety of experimentally induced dominant-negative mutations in cultured cells and transgenic mice causes the disruption of keratin filaments. However depending on the type and the level of expression, large aggregates of resulting aberrant keratin proteins could accumulate and, in some cases, may cause cell toxicity. A careful analysis, however, showed that the phenotype observed with transgenic mice expressing K14 dominant-negative mutations was specific. Some dominant-negative kera-

tin 14 mutations mildly disrupted the filament network without unspecific accumulation of aberrant and insoluble proteins and still perturbed the skin integrity (Coulombe et al. 1991a). The future introduction of targeted *mK8* dominant-negative mutations, corresponding to the skin keratin point mutations will ensure a similar controlled level of mutated mK8 protein expression and permit a direct comparison of these two types of genetic modifications. It would also permit the direct comparison of the effect of the same mutation in distant keratins.

Material and methods

Cell culture

The D3-ES cell line was originally established by R. Kemler (Doetschman et al. 1987). ES cells were routinely cultured in Dulbecco's modified Eagle medium supplemented with 15% FCS, 0.1 mM β -mercaptoethanol, and 1000 units of h-LIF on gelatin-coated plates. LIF was produced by transfecting the pC10-6R plasmid (kindly provided by J. Heath, University of Oxford, UK) (Moreau et al. 1988; Smith et al. 1988; Williams et al. 1988) into COS cells, and the supernatant was subsequently titrated using ES cell-colony plating efficiency.

Targeting vectors and Southern blot analysis

The K8TV1 targeting vector was described previously (Baribault and Oshima 1991). It contains two arms of homology to the *mK8* gene (Endo A α 1), a 1.8-kb *Xba*I fragment and a 1.2-kb *Sma*I fragment, respectively (Vasseur et al. 1985). The *neo*^r gene (pMC1neopA, Stratagene, La Jolla, CA) replaces part of the first exon, including the ATG translation initiation codon (Semat et al. 1988). The modified HSV-tk gene was added 3' (Mansour et al. 1988), followed by the Bluescript vector (Stratagene). ES cells were electroporated with 40 μ g of *Not*I-linearized targeting vector in 0.7 ml of culture medium using a BTX transfecter 100 at 250 V for 5 msec. Cells were plated at a density of 10⁷ cells/90-mm dish. Twenty-four hours after electroporation, 150 mg/ml of G418 and 2 mM gancyclovir (GanC) were added. One plate was selected without GanC to evaluate the enrichment factor of the GanC selection. This enrichment factor was reproducibly 20-fold. After 10 days under selection, single colonies were picked and grown in duplicate. Screening for the targeting events was performed by PCR analysis using 27- to 29-mer oligonucleotides, one complementary to the *neo*^r gene (5'-CGCACGGGTGTTGGGTCGTTTGTTCGG-3') and one located 3' of the targeting vector homology in the *mK8* gene (5'-GCTGAACAGGCAGAGAACTGATTGGGG-3'). Forty cycles of melting, at 93°C for 1 min., annealing at 57°C, for 30 sec, and synthesis at 72°C for 3 min were performed using 100 ng/reaction of each oligonucleotide: 2 units of *Taq* polymerase (Cetus Corp., Emerville, CA) and crude cell lysate (~10,000 cells/reaction) (Hogan et al. 1986). The first screening was done on pools of eight clones. The single clones contained in pools with the expected PCR products were rescreened for the expected 1.4-kb product. Positive clones were expanded further for Southern blot analysis. Five micrograms of *Xba*I-digested DNA was loaded onto a 0.65% agarose gel and transferred to a Zeta-probe membrane (Bio-Rad Laboratories, Cambridge, MA). The filter was hybridized with the 2.5-kb *Eco*RI pseudogene fragment (Endo A α 2) (Vasseur et al. 1985), which is homologous to the exonic sequences of the *mK8* gene, or with the *Xho*I-*Sal*I fragment from the pMC1neopA plasmid. In some cases, geno-

mic DNA cut with *Xho*I and *Hind*III was hybridized with a *Xho*I-*Xba*I fragment located 5' of the *mK8* first exon (5' probe), or with the EA-R6 *Eco*RI fragment of the *mK8* cDNA (Semat et al. 1988).

Germ-line transmission

Two targeted ES cell clones were injected into C57Bl/6 blastocysts (3.5 days) using standard procedures (Hogan et al. 1986). Briefly, morulae were flushed from the uteri of superovulated females and cultured overnight to blastocysts. ES cells (10–15) were microinjected into the blastocoel of each blastocyst. Groups of eight to nine blastocysts were reimplanted in the uteri of pseudopregnant females. Twenty-four chimeras were obtained from 205 injected blastocysts. Nine male chimeras were mated to non-agouti C57Bl/6 females, and germ-line transmission was scored by the presence of agouti offspring in the F₁ litter. The genotypes of these progenies and the subsequent generations were analyzed by Southern blot analysis of DNA isolated from the yolk sac or the tail of the embryos, or the tail tip of adult mice.

Northern blot analysis

Total RNA from various tissues was isolated with by use of guanidium isothiocyanate. Twenty micrograms of total RNA was separated in each lane of a 1% agarose gel, containing formaldehyde, and then transferred to a Zeta-probe membrane (Bio-Rad Laboratories, Cambridge, MA). The filter was hybridized with a *mK8* cDNA fragment EA-R6 (Semat et al. 1988). The same filter was then rehybridized with the full-length *mK18* cDNA (Singer et al. 1986), the *Xho*I-*Sal*I pMC1neopA fragment, and, finally, to a ribosomal protein L32 gene (Dudov and Perry 1984).

Immunobiochemical and histological analysis

Immunofluorescence staining of blastocysts with TROMA-1 antibodies was performed according to Emerson (1988). At later embryonic stages and in adult mice, tissues were embedded in OTC compound and frozen directly on dry ice. Five-micrometer sections were fixed in 100% methanol for 10 min, rinsed with PBS, and incubated with the respective antibodies. TROMA-1, -2 and -3 monoclonal antibodies are directed against mK8, mK18, and mK19, respectively (Kemler et al. 1981; Boller et al. 1987). In most cases, the secondary rabbit anti-rat IgG conjugated with FITC was preabsorbed against mouse immunoglobulin to minimize the background with endogenous immunoglobulin (Vector Laboratories, Burlingame, CA). This was particularly important when staining adult tissues.

Hematoxylin and eosin staining was performed according to standard histological procedures on sections of paraffin-embedded embryos of adult tissues. Paraffin sections were also used for Wright staining (Mucenski et al. 1991).

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