***Elf5* is essential for early embryogenesis and mammary gland development during pregnancy and lactation**

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***Elf5* is an epithelial-specific ETS factor. Embryos with a null mutation in the *Elf5* gene died before embryonic day 7.5, indicating that *Elf5* is essential during mouse embryogenesis. *Elf5* is also required for proliferation and differentiation of mouse mammary alveolar epithelial cells during pregnancy and lactation. The loss of one functional allele led to complete developmental arrest of the mammary gland in pregnant *Elf5* heterozygous mice. A quantitative mRNA expression study and Western blot analysis revealed that decreased expression of *Elf5* correlated with the downregulation of milk proteins in *Elf5*^{+/-} mammary glands. Mammary gland transplants into Rag^{-/-} mice demonstrated that *Elf5*^{+/-} mammary alveolar buds failed to develop in an *Elf5*^{+/+} mammary fat pad during pregnancy, demonstrating an epithelial cell autonomous defect. *Elf5* expression was reduced in Prolactin receptor (*Prlr*) heterozygous mammary glands, which phenocopy *Elf5*^{+/-} glands, suggesting that *Elf5* and *Prlr* are in the same pathway. Our data demonstrate that *Elf5* is essential for developmental processes in the embryo and in the mammary gland during pregnancy.**

The EMBO Journal (2005) 24, 635–644. doi:10.1038/sj.emboj.7600538; Published online 13 January 2005

Subject Categories: development; differentiation & death

Keywords: ETS; mammary gland; milk protein genes; prolactin; prolactin receptor

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Received: 10 August 2004; accepted: 8 December 2004; published online: 13 January 2005

Introduction

The ETS family of transcription factors now consists of more than 50 members that share a homologous DNA binding domain. These proteins play important roles in development and disease. ETS transcription factors are involved in embryonic development, in hematopoiesis, in immune responses, in development of the skeletal system and differentiation of the small intestine (Bassuk and Leiden, 1997; Ghysdael and Boureux, 1997; Ng *et al.*, 2002; Risteovski *et al.*, 2002, 2004; Wolvetang *et al.*, 2002; Xu *et al.*, 2002). Most ETS genes are expressed in a wide range of cell types, but recently a few that are epithelial-specific have been identified. This group includes *ELF3* (*ESX/ESE-1/JEN/ERT*) (Andreoli *et al.*, 1997; Chang *et al.*, 1997; Oettgen *et al.*, 1997; Tymms *et al.*, 1997; Choi *et al.*, 1998), *EHF* (*ESE-3*) (Bochert *et al.*, 1998; Kas *et al.*, 2000), *PDEF* (*PSE*) (Oettgen *et al.*, 2000; Yamada *et al.*, 2000) and *ELF5* (*ESE-2*) (Zhou *et al.*, 1998; Oettgen *et al.*, 1999). *ELF5*, *ELF3* and *ESE-3* share considerable sequence similarity and are all expressed in a similar subset of epithelial tissues.

Elf3 is the most broadly expressed of the epithelial-specific ETS genes. It is evident in the epithelium of the gastrointestinal tract, the mammary gland, uterus and prostate, and the tongue (Andreoli *et al.*, 1997; Chang *et al.*, 1997; Oettgen *et al.*, 1997; Tymms *et al.*, 1997). *Elf5* and *Ese-3* have an overlapping but more restricted pattern of expression compared to *Elf3*. These genes are expressed in a subset of organs containing glandular or secretory epithelium (Oettgen *et al.*, 1999; Kas *et al.*, 2000; Lapinskas *et al.*, 2004). Although there is considerable organ overlap in the expression of *Elf5*, *Elf3* and *Ese-3*, several differences in their expression patterns have been observed. For instance, *Elf3* is expressed strongly in the small intestine and liver, whereas *Elf5* and *Ese-3* do not appear to be expressed in these organs at all. Both *Elf3* and *Ese-3* are expressed in the colon and pancreas, *Elf5* is not, and *Elf3* and *Elf5* are present in the stomach, whereas *Ese-3* is not.

We have previously shown that *Elf5* functions as a transcription factor with similar sequence-specific DNA binding characteristics to other ETS family members (Zhou *et al.*, 1998). *ELF5* (*ESE-2a*) is capable of transactivating a number of epithelial-specific gene promoters, including *SPRR2A*, *PSP* and *PSA*, *in vitro* (Oettgen *et al.*, 1999). In addition, recombinant *ELF5* is capable of transactivating the whey acidic protein (*WAP*) promoter (Thomas *et al.*, 2000) in mouse mammary epithelial cells. This, together with its expression in the mammary gland (Zhou *et al.*, 1998), implies a function for *Elf5* in this organ. In order to establish the biological function of the *Elf5* gene in the mouse and to gain an understanding of the *ELF5* gene in human biology, we generated and characterized *Elf5* null mice.

Results

Elf5 is essential for early mouse embryogenesis

We generated a targeting construct in which part of exon 3 of the *Elf5* gene was replaced by an NLS-LacZ-neo cassette (Figure 1A). The targeting construct was designed to interrupt exon 3, which contains the ATG initiation codon of the *Elf5* protein, and produce a fusion product containing the first 28 amino acids of the *Elf5* protein fused to β -galactosidase. This targeting construct was electroporated into isogenic 129SvJ J1 embryonic stem (ES) cells, and the correctly targeted *Elf5*^{+/-} ES cell clones #1 and #130 were microinjected into C57Bl/6J murine blastocysts. The resulting male chimeras were then mated with *Elf5*^{+/+} C57Bl/6J female mice to generate *Elf5*^{+/-} mice. Genomic Southern blot analysis and PCR screening were used to confirm the correct targeting event (Figure 1B–D). Southern analysis of tail DNA revealed a polymorphism in the wild-type *Elf5* gene locus between 129SvJ and C57Bl/6J genetic backgrounds (Figure 1C).

Heterozygotes were intercrossed to produce 513 offspring derived from the ES cell clone #130. Genotyping revealed that 34.9% were *Elf5*^{+/+} and 65.1% were *Elf5*^{+/-}. No *Elf5*^{-/-} mice were detected (Figure 2A). Similar results were obtained for 43 adult mice derived from the ES cell clone #1 (Figure 2A). These data indicated that the *Elf5*^{-/-} genotype was embryonic lethal.

Since *Elf5*^{-/-} animals were not detected among 3-week-old offspring from heterozygote intercrosses, we investigated the genotypes of neonates and embryos. *Elf5*^{-/-} animals were not detected among neonates nor among embryos at E13.5, E14.5 and E18.5 by Southern blot analysis. PCR analysis of postimplantation embryos dissected from the decidua at E7.5–11.5 (with the morning of vaginal plug detection corresponding to E0.5) showed that none of these embryos were *Elf5*^{-/-} (Figure 2A). These results suggested that the lethality of *Elf5*^{-/-} embryos was occurring before E7.5. Genomic PCR analysis of 58 blastocysts at E3.5, collected from intercrossed *Elf5*^{+/-} females, detected *Elf5*^{-/-} blastocysts in a ratio expected for a Mendelian distribution (Figure 2A and B). These results indicated that *Elf5*^{-/-} embryos die between E3.5 and E7.5, during the period of implantation.

Elf5 is expressed in the pregnant and lactating mammary gland

Our earlier studies (Zhou *et al*, 1998) had established that the *Elf5* transcript was expressed in the mammary gland, but we

Figure 1 Gene targeting of the murine *Elf5* locus. (A) A schematic representation of the targeting strategy. The homologous recombination event replaces a portion of exon 3 and intron 3 with an NLS-LacZ-neo cassette. Black box: exon 3 containing the ATG. The thin line represents plasmid sequences. H: *Hind*III; Sa: *Sall*. (B) Representative Southern blot analyses of surviving ES cell clones. A 400 bp 3'-external probe recognizes a 4.6 kb wild-type and an 8.6 kb targeted *Hind*III DNA fragment, and a *neo* probe recognizes an 8.6 kb targeted *Hind*III DNA fragment. (C) Germline transmission of the targeted *Elf5* allele. A 3'-external probe recognizes 8.6 kb targeted (129SvJ), 4.6 kb wild-type (129SvJ) and 2.0 kb wild-type (C57Bl/6J) *Hind*III bands. Left panel: Southern analysis of an entire litter from Chimera $\times$ C57Bl/6J; right panel: Southern analysis of an entire litter from *Elf5*^{+/-} intercrosses. (D) PCR analysis of progeny from *Elf5*^{+/-} intercrosses detects a 169 bp wild-type and a 237 bp mutant band.

Embryonic stage	No. of litters	Total no. of mice	Genotypes		
			Number of embryos/mice (percentage of total)		
			+/+	+/-	-/-
Derived from ES clone #130					
E3.5	9	58	14 (24.1)	26 (44.8)	18 (31.0)
E7.5	4	40	15 (37.5)	25 (62.5)	0
E8.5	3	18	6 (33.3)	12 (66.7)	0
E9.5	4	36	8 (22.2)	28 (77.8)	0
E10.5	6	46	17 (37.0)	29 (63.0)	0
E11.5	7	45	14 (31.1)	31 (68.9)	0
E13.5	1	6	2 (33.3)	4 (66.7)	0
E14.5	1	6	2 (33.3)	4 (66.7)	0
E18.5	4	33	12 (36.4)	21 (63.6)	0
Neonate	4	24	7 (29.2)	17 (70.8)	0
Adult	77	513	179 (34.9)	334 (65.1)	0
Derived from ES clone #1					
Adult	8	43	13 (30.2)	30 (69.8)	0

Figure 2 *Elf5*^{-/-} is embryonic lethal. (A) Genotype distribution of *Elf5*^{+/-} intercrosses. (B) Genotyping blastocysts. PCR analysis detects a 169 bp wild-type (WT) band and a 237 bp mutant band (KO).

Figure 3 *Elf5* expression in *Elf5*^{+/-} and *Elf5*^{+/+} mammary glands. (A) Northern blot of total RNA from wild-type mammary glands probed with a murine *Elf5* cDNA (top panel) and *18s* cDNA as a loading control (lower panel). (B) Northern blot of poly(A)⁺ mRNA probed with a murine *Elf5* cDNA (top panel) and *Gapdh* cDNA (lower panel). Relative expression levels of *Elf5-b/Gapdh* are indicated (right panel). Lane 1: day 18.5 pregnant *Elf5*^{+/+} mammary gland; lane 2: day 18.5 pregnant *Elf5*^{+/-} mammary gland; lane 3: day 1 postpartum *Elf5*^{+/+} mammary gland; lane 4: day 1 postpartum *Elf5*^{+/-} mammary gland. Band intensities were quantified on a phosphorimager. (C) Western blot of *Elf5* protein in the wild-type and heterozygous glands at days 6.5–14.5 of pregnancy. *Elf5* protein is not visible in the 10.5-day wild-type sample on the right-hand panel because a shorter exposure time is shown.

had not examined *Elf5* expression in this tissue during late pregnancy and lactation. We have now investigated the temporal expression pattern of *Elf5* mRNA in pregnant and day 1 postpartum mammary glands from both *Elf5*^{+/+} and *Elf5*^{+/-} mice. Northern blots of poly(A)⁺ mRNA were probed with the mouse *Elf5* cDNA and the two *Elf5* transcripts, *Elf5-a* (2.5 kb) and *Elf5-b* (1.5 kb), were observed in both pregnant and day 1 postpartum mammary glands from wild-type and heterozygous mice (Figure 3A and B). It should be noted that we did not detect any spurious *Elf5* transcripts on our Northern blots when we compared *Elf5*^{+/+} and *Elf5*^{+/-} mammary gland RNA, indicating that the null allele did not produce detectable alternatively spliced *Elf5* messages. Previously, we showed that *Elf5* mRNA levels sharply increased between days 2 and 10 of pregnancy (Zhou *et al*, 1998). Here we show that this surge of *Elf5* mRNA occurs after day 8, peaks at day 12 and remains at a high level throughout pregnancy and early lactation (Figure 3A). *Elf5*

mRNA levels in the heterozygous pregnant and day 1 postpartum mammary gland were reduced compared to that in the corresponding *Elf5*^{+/+} organs, as expected (Figure 3B). We also measured *Elf5* protein levels and found that *Elf5* protein was present but barely detectable before day 10.5 of pregnancy in the wild-type mammary gland (Figure 3C). At day 12.5, there was a dramatic increase in the amount of *Elf5* protein, consistent with the increase in *Elf5* message at day 12. Surprisingly, *Elf5* protein levels in the heterozygous mammary gland were more dramatically reduced than the message, with no protein detectable (Figure 3C).

Elf5 is essential for pregnancy-associated mammary gland development

Elf5^{+/-} females derived from ES cell clone #130 were fertile and gave birth to litters of morphologically normal pups whose numbers were comparable to those of *Elf5*^{+/+} females. However, despite normal nursing and mothering characteristics displayed by *Elf5*^{+/-} females, most of their first litter pups died within 24 h of birth, and virtually the entire litter had perished by 48 h. This phenomenon was never observed in our wild-type colonies where only the occasional loss of a maximum of one to two pups per litter was observed. All pups were observed to attach to the nipple and suck, but newborns of *Elf5*^{+/-} females died of starvation and dehydration. Lethality of the newborns was independent of their genotype (*Elf5*^{+/+} or *Elf5*^{+/-}). Examination of 1-day-old pups revealed that they lacked milk in their stomachs. These results suggested that *Elf5*^{+/-} females failed to lactate.

To quantify this effect, we studied 49 *Elf5*^{+/-} mothers. Of these, 41 (83.7%) failed to support any pups, three (6.1%) were able to support a reduced number of pups after some had died, and in five cases (10.2%) all the pups born were supported. Two *Elf5*^{+/-} females derived from ES cell clone #1 were also observed for their lactating abilities. Both females failed to lactate after their first pregnancy. A small number of heterozygous females were able to suckle pups successfully. These mice were on a 129SvJ-C57Bl/6J mixed genetic background and this variable phenotype was most probably due to a genetic modifier present in one of the genetic backgrounds. *Elf5*^{+/-} mice were subsequently generated on a pure 129Svter genetic background, and six *Elf5*^{+/-} females were mated with either *Elf5*^{+/+} or *Elf5*^{+/-} males. All six females were incapable of lactation after multiple pregnancies (up to four pregnancies), suggesting that the mammary alveolar developmental defect was 100% penetrant in the 129Svter strain.

Examination of whole mount mammary glands from age-matched virgin *Elf5*^{+/+} and *Elf5*^{+/-} females demonstrated that branching morphogenesis (Hovey *et al*, 2002) was not affected, as elongation and extension of the mammary ductal tree as well as ductal side branching were comparable between genotypes. Alveolar buds formed at the ductal termini with age in both genotypes (Figure 4A–F). Observations made at day 18.5 of pregnancy and 1 day postpartum showed that *Elf5*^{+/-} glands entered the proliferation phase of alveolar morphogenesis (Neville *et al*, 2002), but then development stalled. The degree of retarded alveolar genesis was variable among individuals. In some cases, development stalled at a very early stage of alveolar proliferation and

Figure 4 Mammary gland development is defective in pregnant and postpartum *Elf5*^{+/-} females. Whole-mount analyses of inguinal mammary glands from wild-type (left) and *Elf5*^{+/-} (right) mice. Shown are virgin mice at 48 days (A, B), 131 days (C, D) and 176 days (E, F), 18.5-day pregnant glands (G, H) and day 1 postpartum glands (I, J).

only very few rudimentary alveoli are formed (Figure 4J), whereas in others, development stalled later and alveoli are seen (Figure 4H). However, differentiation and expan-

sion of the alveolar buds into mature lobuloalveolar mammary tissues was severely impaired in *Elf5*^{+/-} females (Figure 4H and J).

We examined the proliferation status of the mammary glands from virgin, pregnant and lactating mice by PCNA staining. The virgin mammary glands of *Elf5*^{+/+} and *Elf5*^{+/-} mice showed no difference in the proliferation of the mammary epithelial cells (data not shown). Likewise, no difference was observed between day 6.5 pregnant mammary glands (Figure 5A, B and Q). However, a lower percentage of proliferating mammary epithelial cells was found consistently in day 8.5 (Figure 5C, D and Q) and 10.5 (Figure 5E, F and Q) pregnant *Elf5*^{+/-} mammary glands compared to wild type. The difference in the percentage of the proliferating mammary epithelial cells between the *Elf5*^{+/+} and *Elf5*^{+/-} mammary glands becomes less evident from day 12.5 to day 18.5 of pregnancy (Figure 5G–N and Q). However, there were fewer proliferating cells in day 1 lactating *Elf5*^{+/-} mammary glands than in the wild-type control (Figure 5O–Q). From the images shown in Figure 5 at lower magnification, it is obvious that the epithelial content of the heterozygous mammary gland is reduced. We also examined the levels of apoptosis in the mammary glands at all the developmental stages mentioned above. The number of apoptotic cells was negligible in all sections tested and no difference was observed between wild-type and heterozygous mice (data not shown).

Taken together, the results in Figures 4 and 5 indicate that the *Elf5*^{+/-} mammary gland defect occurred during pregnancy at the proliferative phase of alveolar morphogenesis and that this is a direct result of a partially impaired proliferation of mammary epithelial cells, and not due to increased apoptosis. Notably in the wild type, histology shows that the alveoli subunits expanded and filled with milk, indicating the functional secretory state of the mammary gland (Figure 5M and O). In contrast, there was no expansion of the alveolar lumens in *Elf5*^{+/-} mammary glands in which alveoli had formed. These observations indicate that in individuals where alveolar development proceeded past the proliferative phase, it was blocked during the secretory initiation phase (Figure 5N and P).

The *Elf5*^{+/-} mammary defect is epithelial cell autonomous

In order to determine if the defect in mammary gland development observed in *Elf5* heterozygous females was due to the lack of *Elf5* in the mammary epithelium *per se* or whether the defect was secondary due to defects in other endocrine systems, we transplanted wild-type and *Elf5*^{+/-} mammary epithelium (Naylor *et al*, 2003) into *Rag1*^{-/-} recipient females (Mombaerts *et al*, 1992), which cannot reject transplants due to their compromised immune systems. These females were then mated. The wild-type transplants displayed ductal branching and alveolar proliferation comparable to unmanipulated wild-type glands (Figure 6A and B). The *Elf5* heterozygous glands showed side branching but no lobuloalveolar development (Figure 6C and D), demonstrating that the *Elf5*^{+/-} mammary defect was epithelial cell autonomous and not dependent on other factors.

Gene expression profiles in *Elf5* and *Prlr* heterozygous mammary glands

The observation that a similar mammary gland developmental defect also occurs in *Prlr*^{+/-} mice (Ormandy *et al*, 1997) prompted us to examine the levels of *Elf5* in the mammary

glands of *Prlr*^{+/-} females. A quantitative PCR analysis demonstrated that *Elf5* mRNA was significantly reduced in the *Prlr*^{+/-} day 1 postpartum mammary glands compared to wild-type controls (*t*-test, *P*=0.0157) (Figure 7A). On the contrary, *Prlr* expression was unchanged in *Elf5*^{+/-} glands compared to wild types, indicating that *Elf5* is downstream in the *Prl* signalling pathway (Figure 7B). We also used quantitative PCR to examine the expression levels of a panel of genes encoding milk proteins in the *Elf5*^{+/-} glands. *WDM1/EXP1*, *WAP*, *CEL* and β -casein were all reduced in the heterozygous glands compared to wild-type controls (Figure 7C). Furthermore, Western blot analysis of the milk proteins α -casein, β -casein and WAP in *Elf5*^{+/-} mammary gland extracts revealed that these milk proteins were not synthesized in the heterozygous gland (Figure 7D).

Discussion

We demonstrate that *Elf5* is an ETS factor required for early mouse development. A number of ETS transcription factors play essential roles in mouse embryogenesis. Of the 10 ETS family members that have been disrupted in mice, *Pu.1*^{-/-}, *Tel*^{-/-}, *Flt1*^{-/-}, *Elf3*^{-/-} and *Gabpa*^{-/-} were embryonic lethal around E16.5, E10.5–11.5, E11–12.5, E11.5 and prior to implantation, respectively (Scott *et al*, 1994; Wang *et al*, 1997; Hart *et al*, 2000; Spyropoulos *et al*, 2000; Ng *et al*, 2002; Ristevski *et al*, 2004). In contrast, *Ets2*^{-/-} embryos died before E8.5 due to growth arrest of the ectoplacental cone and apoptosis of the embryonic ectoderm (Yamamoto *et al*, 1998). Breakdown in the vascular interactions between the embryo and the maternal circulation also contributed to the *Ets2*^{-/-} embryonic lethality (Yamamoto *et al*, 1998). In this regard, it is important to note that while the ETS proteins all share a similar DNA binding domain structure, have similar cognate DNA binding elements and in many cases overlapping expression, there is no functional redundancy during development.

Although the status of *Elf5* expression in the early mouse embryo and its corresponding extraembryonic tissues (before E9.5) is unknown, *Elf5* was found to be expressed in the placenta throughout E9.5–18, indicating a potential function for *Elf5* in this tissue (Zhou *et al*, 1998). The placenta is derived from the trophoblast (Kaufman and Bard, 1999), and the trophoblast of the blastocyst is important for the exchange of metabolites between the mother and the embryo. Therefore, the early lethality of *Elf5*^{-/-} embryos may be due to the loss of *Elf5* function in the embryonic trophoblast. However, we cannot rule out possible defects in the inner cell mass of the developing embryo.

Although it may be argued that the phenotype observed is due to the dominant behavior of a truncated *Elf5* protein generated from the targeted allele, we believe that this is extremely unlikely. The disruption in the *Elf5* gene occurs 28 amino acids after the initiating methionine and if splicing occurred from the truncated exon 3 (skipping the *LacZ-neo* cassette) through to exons 4, 5 or 6, there would be a frameshift such that no functional domains of the *Elf5* protein would be produced. Also, we do not see any unexpected *Elf5* transcripts in Northern blots performed on our heterozygous mice. Unfortunately, we were unable to extract sufficient quantities of high-quality RNA from the *Elf5*^{-/-} blastocysts for Northern blot analysis.

Figure 5 Histological analyses and PCNA staining of wild-type and *Elf5* heterozygous pregnant mammary glands. Left two panels: wild type; right two panels: *Elf5*^{+/-}. Low-magnification images (A–P) show under development of *Elf5*^{+/-} glands. Day 6.5 (A, B); day 8.5 (C, D); day 10.5 (E, F); day 12.5 (G, H); day 14.5 (I, J); day 16.5 (K, L); day 18.5 (M, N); day 1 lactation (O, P). PCNA staining is shown as a brown nuclear precipitate in epithelial cells (A–P). The percentage of PCNA-positive cells throughout pregnancy-associated mammary gland development is shown (Q). Results are the average of four sections per mouse, *n* = 2/3, error bars represent $\pm$ s.e.m.

Figure 6 Whole-mount analysis of wild-type and *Elf5* heterozygous mammary epithelium transplanted to a normal host mammary fat pad. Wild-type (A, B) and *Elf5*^{+/-} (C, D) glands are shown. (A, C) Whole mount; (B, D) histology sections.

A number of epithelial-specific ETS transcription factors (ELF3, ELF5 and ESE-3) are expressed in the mammary gland, suggesting potential functions for these proteins there (Chang *et al*, 1997; Neve *et al*, 1998; Oettgen *et al*, 1999; Kas *et al*, 2000). We have shown that both ELF3 and ELF5 can transactivate a pregnancy- and lactation-associated milk protein (*WAP*) gene promoter *in vitro* (Thomas *et al*, 2000). However, neither Elf3 nor ESE-3 is able to compensate for the lack of Elf5 in the mouse mammary gland. In this study, we have demonstrated that *Elf5* has a specific, non-redundant role in mammary gland development during pregnancy and lactation. Furthermore, the phenotype is observed in the heterozygous state but intriguingly, even though we can detect *Elf5* message in the heterozygous glands, the levels of Elf5 protein are reduced more than expected. Indeed, the protein is undetectable on our Western blots. With the exceptions of *Prlr*^{+/-}, *Plg*^{+/-} (*plasminogen*) and *Ptc1*^{+/-} (*patched-1*) females (Ormandy *et al*, 1997; Lewis *et al*, 1999; Lund *et al*, 2000), no other gene identified as being involved in mammary gland development and function displayed a phenotype in the heterozygous state and this phenomenon has never been observed with any other ETS factor.

The mammary ductal tree can elongate and branch quickly through the entire mammary fat pad under the stimulation of ovarian hormones following puberty (reviewed in Hovey *et al*, 2002). Pregnancy hormones then promote alveolar proliferation, and the alveolar epithelial cells from the resulting lobuloalveolar structures eventually differentiate into secretory epithelial cells at parturition (reviewed in Neville *et al*, 2002). Our results demonstrate that mammary epithelial cell proliferation and differentiation during pregnancy and the postpartum period depends on the expression of *Elf5*, and that one functional *Elf5* allele is not sufficient for function. In contrast, the earlier developmental events of ductal morpho-

genesis do not show this sensitivity to reduced *Elf5*. Death of the pups born to *Elf5*^{+/-} females was due to a failure of mammary lobuloalveolar development and the consequent failure in maternal milk production. Thus, *Elf5* has a dual function in the mammary gland. It is required for proliferation in early pregnancy, then for differentiation later on. Notably, a significant increase in *Elf5* protein was observed during mid-pregnancy (at day 12.5) when the mammary alveolar epithelial cells start to undergo secretory initiation, suggesting that *Elf5* expression may trigger the onset of lactogenesis of the mammary alveolar epithelial cells during pregnancy. The expression of *WDM1/EXP1* and β -casein begins around day 9 of pregnancy and that of *WAP* at day 14 (Robinson *et al*, 1995; Kannius-Janson *et al*, 1998), coinciding with the upsurge in *Elf5* expression. Thus, an apparent correlation exists between the onset of milk protein gene-associated differentiation of the wild-type mammary alveolar epithelial cells and the expression levels of *Elf5*. Our data suggest that in the *Elf5* heterozygous mammary gland, the level of *Elf5* is insufficient to trigger the expression of genes required for differentiation of the epithelial cells and subsequent lactogenesis. In effect, what we observe at day 18.5 of pregnancy is cells blocked at an earlier stage of differentiation.

The mammary defect in *Elf5*^{+/-} females closely resembled that in *Prlr*^{+/-} females where an impaired differentiation of lobuloalveolar units resulted in an inability to lactate (Ormandy *et al*, 1997). Since disruptions in either *Elf5* or *Prlr* produce a similar phenotype, it is likely that *Elf5* and *Prlr* participate in the same pathway. This study and the one by Naylor *et al* (2003) support that a possible downstream link for *Elf5* is in the prolactin signalling pathway.

Quantitative RT-PCR showed that *Elf5* expression is down-regulated in the *Prlr*^{+/-} day 1 postpartum mammary gland

Figure 7 Gene expression analysis in *Elf5* and *Prlr* heterozygous mammary glands. (A) *Elf5* mRNA expression (isoforms a and b) in *Prlr*^{+/+} versus *Prlr*^{+/-} glands. Results are shown as the average number of *Elf5* transcripts (*Prlr*^{+/+}: *n* = 4, *Prlr*^{+/-}: *n* = 3). A statistical *t*-test was performed between the two groups. (B) *Prlr* mRNA expression in *Elf5*^{+/+} versus *Elf5*^{+/-} glands. There is no significant difference between wild types and heterozygotes. (C) Milk protein gene expression in *Elf5*^{+/-} versus *Elf5*^{+/+} glands. The genes tested are as indicated (CSNβ is β-casein). (D) Milk protein expression in *Elf5*^{+/-} versus *Elf5*^{+/+} glands.

and that *Prlr* levels are unchanged in *Elf5*^{+/-} glands, placing *Elf5* downstream in the Prl/*Prlr* signalling cascade. Significantly, the expression of four genes encoding the

milk proteins WDNM1/EXP1, β-casein, CEL and WAP is downregulated. Our data demonstrate that these genes are all regulated, either directly or indirectly, by *Elf5*; thus, it is likely that the mammary gland developmental defect observed in the *Elf5*^{+/-} pregnant female is due to a block in milk protein-associated alveolar differentiation.

The action of prolactin on *Elf5* gene expression is most likely mediated via the JAK2/STAT or MAP kinase signalling pathways. Stat5 is activated by tyrosine phosphorylation in response to Prl stimulation. *Stat5a* null mice exhibit defects in lobuloalveolar proliferation and differentiation (Liu *et al*, 1997); therefore, it is possible that Stat5a regulates expression of *Elf5*. Interestingly, the *Elf5* promoter has a putative Stat binding site but this site is yet to be proven functional. Other ETS factors are known to be targets of MAP kinases (Graves and Petersen, 1998), but the conserved MAP kinase phosphorylation site present in ETS1, ETS2 and pntp2 (Yang *et al*, 1996) is not present in *Elf5* (Zhou *et al*, 1998). Other ligand/receptor pathways may also involve *Elf5*. For instance, mouse knockouts of the ligand OPGL and its receptor RANK (Fata *et al*, 2000) and of the LAR receptor-like protein tyrosine phosphatase (Schaapveld *et al*, 1997) all produce mammary gland defects characterized by a failure of alveolar terminal differentiation similar to that of *Elf5*^{+/-} female mice, but the involvement of *Elf5* in these pathways has yet to be tested.

Finally, our transplant studies strongly suggested that the mammary gland defect in the *Elf5*^{+/-} mice is epithelial cell autonomous and not due to other influences such as systemic endocrine alterations since the *Elf5*^{+/-} mammary epithelium transplanted to a wild-type mammary fat pad failed to undergo pregnancy-associated lobuloalveolar development.

Epithelial cell proliferation and differentiation is tightly controlled during mammalian development. This study has provided *in vivo* evidence of the involvement of *Elf5* in these critical developmental processes and further defined a dual role for *Elf5*. It is initially required for early embryonic development and again later in life during development of the mammary gland in pregnancy. The role of *Elf5* in the mammary gland is extremely interesting since in the absence of one *Elf5* allele, the development of the gland during pregnancy is completely shut down. How *Elf5* exerts its effects on the processes of proliferation and differentiation in the mammary gland is the subject of an ongoing study and has important implications for both development and diseases such as breast cancer.

Materials and methods

Gene targeting

Murine *Elf5* genomic clones were isolated from a 129SvJ λFIXII genomic library (Stratagene) using an m*Elf5* cDNA probe. The targeting construct contained a 2.2 kb fragment, encoding the first 84 bp of m*Elf5* cDNA coding sequence as the left arm and a 1.65 kb *Sall*-*Bam*HI fragment as the right arm. The homologous recombination event replaces a portion of exon 3 and intron 3 with an NLS-*LacZ*-*neo* cassette in which the pMC1-*neo* cassette was in a reverse orientation to that of *Elf5* transcription. A pMC1-TK cassette was placed 3' of the right arm to allow for enrichment of targeted ES cells. *Hind*III-digested genomic DNA was used to determine the genotype by Southern blot analysis using a 400 bp probe as indicated in Figure 1. PCR genotyping used two m*Elf5*-specific primers, P_S (5'-GCACACCCAGAATTGAAGATTCC-3') and P_{AS1} (5'-CC TTCCTGACCTGGACTG-3'), and one *neo*-specific primer P_{NEO} (5'-ATTCGCCAATGACAAGACGC-3').

Mammary gland whole mounts and histology

For whole mounts, inguinal mammary glands were fixed in Carnoy's fixative and stained in carmine alum as described (Kordon *et al*, 1995). For histology, inguinal mammary glands were fixed in Bouin's fixative and paraffin sections (10 µm) were stained with hematoxylin and eosin.

Western blots

Protein was extracted from thoracic mammary glands removed at different stages of pregnancy using Trizol (Invitrogen). The protein extracts were resolved on a 12% SDS-PAGE gel and then transferred to Immobilon PVDF membrane (Millipore). After blocking, membranes were incubated with goat anti-Elf5 (N-20) antibody (Santa Cruz) at a 1:100 dilution. Secondary antibody was a 1:4000 dilution of horseradish peroxidase-conjugated anti-goat immunoglobulin (DAKO) incubated at room temperature for 1 h. SuperSignal West Pico Chemiluminescent Substrate (Pierce) was used to visualize binding after exposure of the membrane to CLX-Posure film (Progen). To control for protein loading and epithelial content, the membrane was washed and then incubated with a mouse anti-cytokeratin 5/8 monoclonal antibody (BD Pharmingen) at a 1:1000 dilution for 1 h at room temperature. Signal was visualized by incubating with a 1:2000 dilution of horseradish peroxidase-conjugated anti-mouse immunoglobulin (DAKO). To determine milk protein expression, the membrane was stripped by washing three times in 50 mM glycine pH 2.0, then washed once in TBST and incubated with a 1:10 000 dilution of polyclonal rabbit antisera for milk-specific proteins (Accurate Chemical and Scientific Corporation). Horseradish peroxidase-conjugated anti-rabbit immunoglobulin (DAKO) was used at a 1:2000 dilution and signal visualized as described above.

PCNA and TUNEL staining

After fixation in Bouin's fixative for 4 h at room temperature, inguinal mammary gland tissue from *Elf5*^{+/+} and *Elf5*^{+/-} females was embedded in paraffin and sectioned at 5 µm. Sections were cleared in histosol and rehydrated. To detect apoptosis, the ApopTag® Peroxidase *In situ* Apoptosis Detection Kit (Serological Corporation) was used essentially according to the manufacturer's instructions, except no proteinase K pretreatment was required. To detect cell proliferation, antigen retrieval was performed by heat treatment in 10 mM sodium citrate buffer (pH 6.0). Endogenous peroxidase activity was quenched using 6% (v/v) H₂O₂ in methanol for 30 min at room temperature. Tissue sections were blocked using CAS Block (Zymed Laboratories Inc.) for 40 min at room temperature. Sections were immunoreacted with (neat) mouse monoclonal antiproliferating cell nuclear antigen antibody EPOS anti-PCNA/HRP, clone PC10 (DAKO) or the EPOS Immunoglobulins/HRP Negative Control (DAKO) for 60 min at room temperature. Immunoperoxidase staining was detected using the Liquid DAB Substrate-Chromogen

System (DAKO). All sections were counterstained lightly with Mayer's hematoxylin.

Quantification of cellular proliferation and apoptosis

Images from PCNA and TUNEL reacted mammary gland consecutive sections were captured with a CoolSNAP-Pro color camera attached to an Olympus BX60 microscope with Image-Pro Plus software. The total number of cells and the number of proliferating or apoptotic cells in four representative fields from each mouse were enumerated.

Mammary epithelium transplants

Transplants were performed as described (Briskin *et al*, 1999). Mammary gland fragments from 16-week-old *Elf5* heterozygotes and wild-type littermates were transplanted into the cleared fat pads of 3-week-old C57Bl6/Rag1^{-/-} mice (Mombaerts *et al*, 1992). The recipients were mated at 8 weeks post-transplant and the transplants examined by whole-mount microscopy and histology at day 1 postpartum. The whole mounts were stained with carmine alum and the sections stained with hematoxylin and eosin.

Gene expression studies

Poly(A)⁺ mRNA was extracted from the inguinal mammary glands of *Elf5* heterozygotes and wild-type littermates at day 18.5 of pregnancy and at day 1 postpartum. Two samples of each were reverse transcribed using AMV reverse transcriptase (Promega). PCR primers were designed for *WDM1*, *CEL*, *β-casein* and *WAP* using Macvector. To ensure that the reaction was specific for cDNA and not genomic DNA, the primers were designed spanning an intron. PCR primers for *Elf5* were (1) sense primer 5'-TGGACTCCG-TAACCCTAGCACCT-3' (2) antisense primer 5'-ATTGCT-TAAGGGCTGATGGCATCG-3'. This primer pair detects both mRNA isoforms *Elf5a* and *Elf5b* (Zhou *et al*, 1998). The PCR reactions were performed in a LightCycler (Roche) using 1 µl of the cDNA diluted 1:2, 5 pmol of primers and the FastStart DNA master SYBR Green I enzyme mix (Roche) in a 10 µl reaction volume. Relative quantification of the product was performed by comparing the crossing points of different samples above background and assuming that a difference of one cycle in the linear phase of the reaction corresponds to a two-fold difference in transcript levels between samples. The samples were analyzed twice and the results reported as an average of both animal and analysis duplicates. For quantitative analysis of *Prlr* expression, an ABI TaqMan Gene Expression Assay was used.

Acknowledgements

We thank Caroline Wang and Belinda Duscio for excellent technical assistance.

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