

Role of DDC-4/sFRP-4, a secreted Frizzled-related protein, at the onset of apoptosis in mammary involution

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Abstract

Using differential display, we isolated DDC-4, a secreted frizzled-related protein (sFRP), which is induced in the physiological apoptosis of hormonally regulated, reproductive tissues such as mammary gland, prostate, corpus luteum and uterus. The role of this gene in apoptosis was studied in animals overexpressing ectopic DDC-4/sFRP-4. Transgenic mice bearing the DDC-4/sFRP-4 cDNA under the control of the MMTV-LTR promoter showed lactational insufficiency and many apoptotic cells in the alveoli between day 19 of pregnancy and day 4 of lactation as demonstrated by TUNEL reaction and the presence of activated caspase-3. We performed a PKB/Akt kinase assay and studied several of its substrates using phosphorylation-specific antibodies to show reduced phosphorylation in PKB/Akt itself, as well as in glycogen synthetase kinase-3 β (GSK-3 β), BAD, and Forkhead. Taken together, our results show a role for DDC-4/sFRP-4 in abrogating an epithelial cell survival pathway at the onset of mammary gland involution.

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Keywords: sFRP-4; mammary gland; transgenic mice; apoptosis; caspase-3; PK-B/Akt; BAD; Forkhead; GSK-3 β

Abbreviations: FRP, secreted frizzled-related protein; GSK-3 β , glycogen synthetase kinase-3 β ; DDC, differential display coincidence; APC, Adenomatous polyposis coli.

Introduction

The phenomenon of programmed cell death is particularly prominent in the reproductive cycle of the mammary gland. During pregnancy, the mammary epithelium proliferates profusely, but full differentiation is achieved at lactation. Following weaning, the differentiated mammary epithelium, no longer needed, involutes by a process of programmed cell death, returning essentially to the resting state. Our research employs this physiological model for programmed cell death in the mouse, taking advantage of this rapid and synchronous involution of the normal mammary gland to study the processes of programmed cell death. This programmed cell death takes the form of apoptosis.^{1,2}

We were interested in identifying genes involved in the control and execution of mammary gland apoptosis.^{2,3} Since the process of mammary involution is complex, comprising a tissue remodeling program that proceeds in parallel with programmed cell death, a differential display coincidence (DDC) method was employed to recognize genes upregulated in common between two models of physiological apoptosis: the involuting mammary gland and the prostate following castration.³ One of the most interesting genes isolated, *DDC-4*, showed striking homology to the ligand-binding domain of several *frizzled* family, 7-pass transmembrane receptors. *DDC-4* was subsequently recognized as encoding a product belonging to the secreted frizzled-related protein (sFRP) subfamily,⁴ and is referred to here as DDC-4/sFRP-4.

Frizzled proteins are cell surface receptors for Wnt/Wingless (Wg) ligands. In this well-characterized pathway, Wnt/Frizzled signal transduction targets a key cellular regulatory molecule, β -catenin, which is mainly sequestered with E-cadherin at the cell membrane. The small amounts of free β -catenin that occur in the cell are normally degraded in the adenomatous polyposis coli (APC) pathway, thus limiting the availability of β -catenin to act as a cofactor for transcription factors of the Tcf-Lef family.⁵ Wnt/Frizzled signaling acts to brake the degradation of β -catenin, thus increasing levels of the free regulator and activating the Tcf-Lef-dependent transcription of a set of genes including *c-myc*,⁶ *cyclin D1*,^{7,8} and *c-jun*.⁹ Interestingly, hyperactivity of the Wnt/ β -catenin signaling pathway has been linked to several malignancies in man.¹⁰

Whereas the Wnts are a diverse family of insoluble, extracellular matrix-associated proteins, the 7-pass transmembrane Frizzled receptors are both diverse and promiscuous in their specificity for the Wnt ligands.¹¹ The sFRPs^{12,13} are tightly regulated at the transcriptional level¹⁴ and presumably play a modulatory role by competing with the closely related receptor Frizzled for the Wnt ligands. This allows the system to respond rapidly to changing stimuli; the sFRPs can rapidly quench a locally pre-existing signaling field.

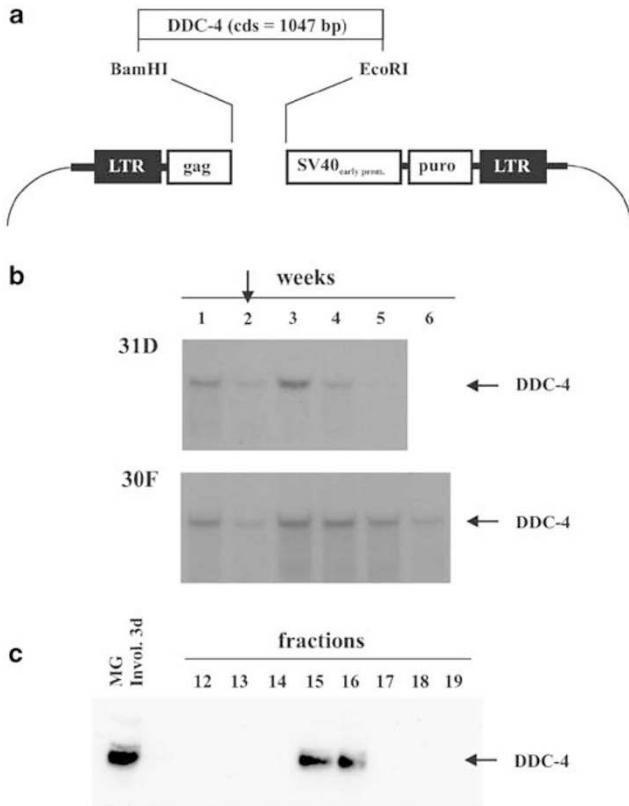


Figure 1 (a) Schematic representation of the *DDC-4* retroviral construct. (b) Northern blot analysis of *DDC-4/sFRP-4* expression in retrovirally infected line 31D and 30F mammary cells after different times in culture. Each track was loaded with 5 μ g total RNA. The arrow indicates the time of the last selection with puromycin. (c) Western blot analysis using affinity-purified, polyclonal rabbit anti-*DDC-4* antibody to detect *DDC-4/sFRP-4* secreted into cell culture supernatants. Peak elution of *DDC-4/sFRP-4* from a heparin-Sepharose affinity column was at 420 mM KCl. A culture supernatant (10 ml) from *DDC-4/sFRP-4*, retrovirally infected clone 30F cells was passed over the column and eluted in 1 ml fractions. MG: extract of 3 day involuting mouse mammary gland as positive control

Since the upregulation of *DDC-4/sFRP-4* has been seen associated with physiological apoptosis in several contexts, the obvious question is whether it is causally involved in initiating apoptosis.

Results

To address this question we have used two different but complementary experimental designs. The first approach consisted of local release of *DDC-4/sFRP-4* protein by implanting *DDC-4/sFRP-4* overexpressing cells into the mammary gland to disturb the local balance between Wnt to Frizzled receptor interaction. The second approach involved the establishment of *DDC-4/sFRP-4* transgenic mice taking advantage of the MMTV-LTR promoter for mammary gland-specific transgene expression.¹⁵

Establishment of cell lines overexpressing *DDC-4/sFRP-4*

For gene transfer of *DDC-4*, we prepared a *DDC-4*-pBabe^{puro} construct containing the full-length coding sequence of the

DDC-4/sFRP-4 secreted *frizzled*-related gene, which has been shown by *in vitro* translation to encode a protein of the expected length.⁴ The retroviral construct is shown schematically in Figure 1a. Using the BOSC ‘helper’ packaging cells,¹⁶ retroviral titres of 2×10^5 infectious units/ml were obtained. The mammary gland-derived cell lines 31D and 30F as well as primary mammary fibroblasts were infected with this construct and selected with puromycin. Northern blot analysis was employed to monitor expression from the *DDC-4/sFRP-4* retroviral construct in the infected cells. The infected epithelial 31D cells exhibited strong expression of *DDC-4/sFRP-4* 1 week after infection. Expression, however, was downregulated already by 3 weeks in culture (Fig 1b). In contrast, retroviral expression of *DDC-4/sFRP-4* was maintained at high levels for up to 6 weeks in the infected fibroblast-like 30F cells (Figure 1b) as well as in primary mammary gland-derived fibroblasts (data not shown). Figure 1c shows a Western blot of cell culture supernatants using affinity-purified, polyclonal rabbit anti-*DDC-4* antibody indicating secretion of the *DDC-4/sFRP-4* protein into the culture media by the infected 30F cells. Culture supernatants were applied to a heparin-Sepharose column and *DDC-4/sFRP-4* was eluted with a salt gradient peaking at 420 mM KCl. In parallel experiments with cell lysates, less amount of *DDC-4/sFRP-4* was found to be cell associated (data not shown). Over an observation time of 8 weeks, we did not observe any phenotypic consequences of retroviral *DDC-4/sFRP-4* expression *in vitro* in any of the infected cells with regard to induction of apoptosis or reduced growth rate (data not shown).

Local overexpression of *DDC-4/sFRP-4* in the mammary gland

We planned to study the effect of *DDC-4/sFRP-4* overexpression using the mammary epithelial reconstitution approach involving removal of the mammary epithelial anlage from normal, 3-week-old mice and replacement with *ex vivo* infected mammary epithelial cells.¹⁷ For this approach, however, transgene expression must be maintained for some weeks. The rapid downregulation of the integrated retroviral expression in the epithelial 31D cells discouraged us from attempting the classical mammary reconstitution experiment with *DDC-4*-infected epithelial cells. We therefore chose to achieve local overexpression of *DDC-4/sFRP-4* by transplanting the more stably expressing infected 30F fibroblast cells into the intact epithelial anlage. The phenotypic effect on the developing endogenous epithelium was studied morphologically and by *in situ* detection of apoptotic cell death (TUNEL). Transplantation of 30F mammary fibroblastic cells expressing *DDC-4/sFRP-4* in the pubertal mammary gland resulted in local, abnormal, dysplastic development of the endogenous epithelium (Figure 2b). Terminal end-buds formed numerous, extremely small bud-like immature alveolar protrusions, but failed to extend normally through the fatty tissue (data not shown). Moreover, the TUNEL assays revealed local apoptotic cell death in the developing mammary epithelium in regions of implanted, *DDC-4/sFRP-4*-expressing 30F cells, but not in regions transplanted with normal 30F cells. In Figure 2c, two TUNEL-positive cells are apparent, as against 26 positive cells shown in the field of Figure 2d.

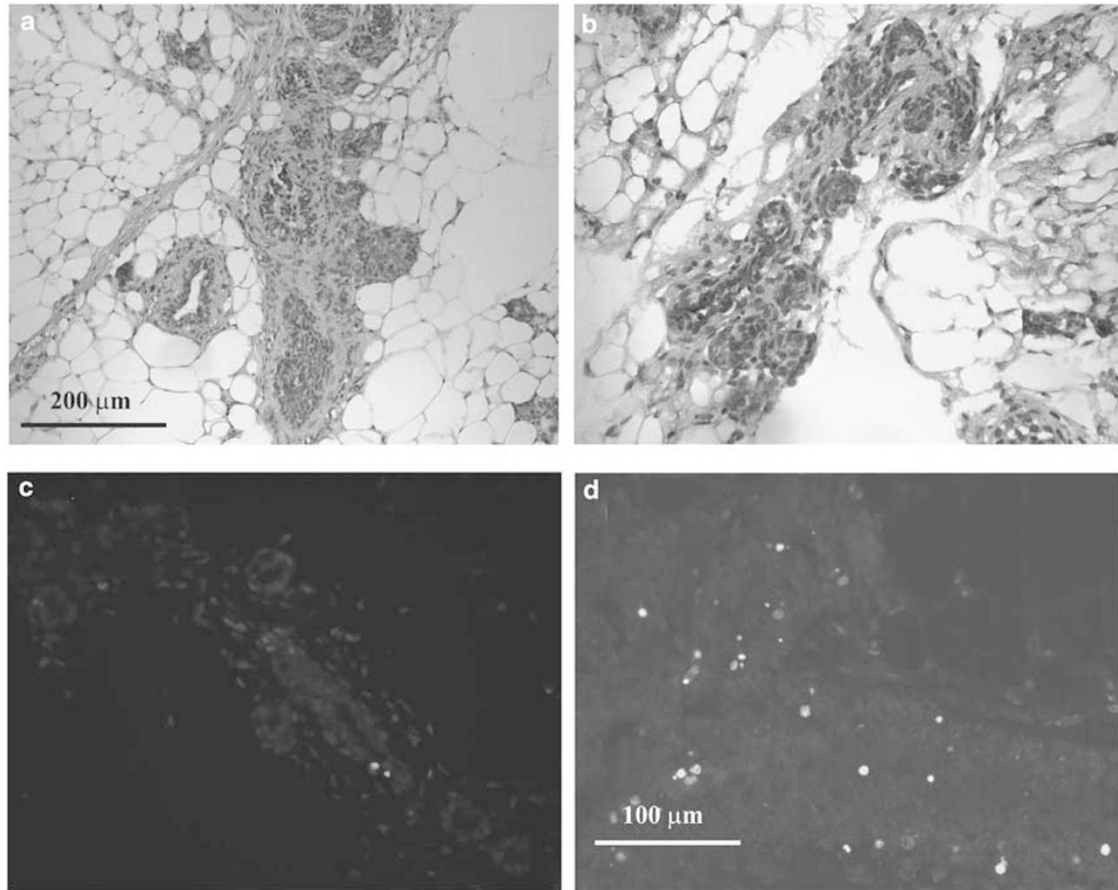


Figure 2 Phenotypic consequences following transplantation of DDC-4-expressing line 30F fibroblastoid cells are illustrated with micrographs of histological sections (a–d). Infected 30F cells were transplanted into the intact mammary gland of pubertal BALB/c mice. Cells infected with retroviral vectors lacking the insert (a, c) are contrasted with cells receiving the DDC-4-pBabe^{puro} (b, d). Hematoxylin and eosin staining is shown in a and b. TUNEL detection of DNA fragmentation employing BODIPY-labeled dUTP is presented in c and d. The magnifications for a, b and c indicated by the bar in a; d was taken at a higher magnification as indicated

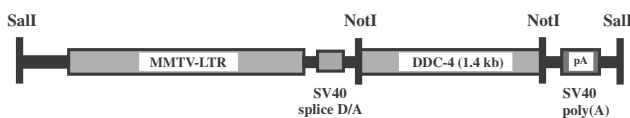


Figure 3 Schematic representation of the MMTV-LTR-DDC-4 transgene

Establishment of DDC-4/sFRP-4 transgenic mice

The mouse mammary tumor virus (MMTV)-LTR has been extensively employed as a promoter in transgenic constructs aiming to achieve tissue-specific mammary expression in a developmental window between late pregnancy and lactation.¹⁸ This time period was particularly appropriate for our study, in that early lactation is a time of differentiated function in the mammary gland; neither proliferation nor apoptosis present themselves as a significant background to interfere with the recognition of a DDC-4/sFRP-4-induced phenotype.

The transgene construct used to produce the transgenic animals is schematically shown in Figure 3. Eight positive transgenic founders were identified based on PCR-DNA analysis; however, only two proved to be *DDC-4/sFRP-4*-expressing lines, 718 and 721. Northern blots were performed

with RNA from the mammary gland obtained at different reproductive stages. As expected for the MMTV-LTR promoter, animals of line 718 showed maximal *DDC-4/sFRP-4* expression in mammary glands at early lactation (Figure 4a). Expression at puberty was totally negative, and, interestingly, transgene expression was not observed during pregnancy before the day 18 (data not shown). In contrast, line 721 exhibited highest levels of *DDC-4/sFRP-4* expression in later lactation, while it was undetectable at late pregnancy (Figure 4a). Strange *et al.*¹ and Guenette *et al.*¹⁹ have established sulfated glycoprotein-2 (SGP-2; clusterin) as an excellent marker for apoptosis in the mammary gland and, thus, its expression was investigated in the DDC-4/sFRP-4 transgenic animals by probing the same blots as discussed above. Interestingly, the levels of *SGP-2* expression observed correlated well with transgene expression (Figure 4a). Western blot analyses of protein extracts from line 718 mammary glands at different stages of development showed that DDC-4/sFRP-4 protein expression was consistent with the RNA levels (Figure 4b).

Both lines expressed the transgene most highly in the largest fourth inguinal mammary gland and less in the second. Tissue specificity was only partial; as previously reported,²⁰

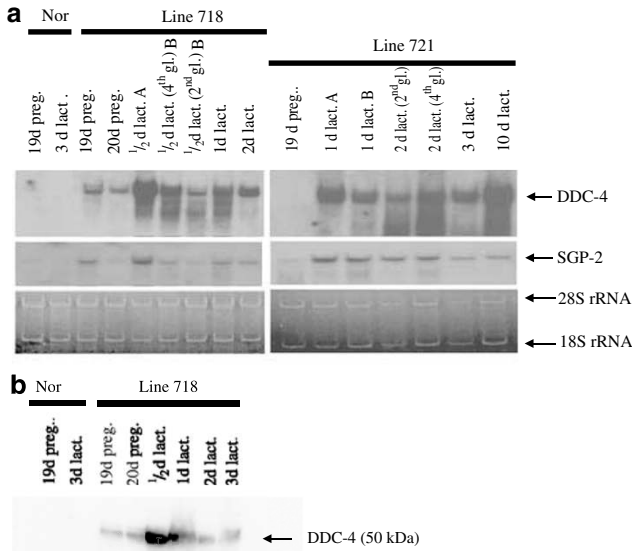


Figure 4 (a) Northern blot analysis of DDC-4/sFRP-4 expression in line 718 and line 721 transgenic mice. RNA was isolated from 19-day pregnant (19d preg.) and 3-day lactating (3d lact.) normal C57BL/6 mice and from the transgenic lines at different times: 19-day pregnant (19d preg.), 20-day pregnant (20d preg.), lactating one-half day, mouse A (1/2 d lact. A), and mouse B (1/2d lact. B), 1 day (1d lact.), 2 days (2d lact.), 3 days (3d lact.) and 10 days (10d lact.). Standard was to sample the fourth mammary gland; two examples are presented, however, comparing the fourth (4th gl.) and second mammary glands (2nd gl.). The blot was probed successively with *DDC-4*, and with sulfated glycoprotein-2 (*SGP-2*). As loading control, the ribosomal RNA bands in an acridine-orange-stained gel are presented. (b) Western blot analysis of DDC-4/sFRP-4 expression in line 718 at various stages of mammary gland development

Table 1 Young-rearing characteristics of transgenic lines 718 and 721 in comparison with normal C57BL/6 mice

Animals	Litter size (no. of litters)	Mean progeny weight at 7 days of age (g)
Line 718	4.8 (5)	2.8 ± 0.2
Line 721	6.3 (3)	3.4 ± 0.2
Normal C57BL/6	9.7 (3)	3.9 ± 0.2

All data displayed refer to first litters of heterozygote DDC-4/sFRP-4-expressing mothers. The indicated litter sizes are averaged over the number of litters indicated

the salivary gland was also an important site of expression in lactating animals (data not shown). Other organs tested were kidney, liver, brain, skin, and testis, which were uniformly negative (data not shown).

The phenotype of transgenic DDC-4/sFRP-4-expressing animals

A comparison of lines 718 and 721 with negative controls in terms of young-rearing characteristics revealed that both lines have reduced litter sizes as well as reduced mean progeny weights at 7 days of age (Table 1). Young from the 718 line fostered from the first day after birth by control mothers

showed normal weight gain at 7 days of age (data not shown). This indicates that the DDC-4/sFRP-4 transgenic animals exhibit a lactational defect, at least at the beginning of the lactating period.

In order to document the phenotype of DDC-4/sFRP-4 overexpression in the mammary gland, we examined the tissue samples with a variety of histological procedures. Figure 5a and b show the morphology at 12 h postpartum after hematoxylin and eosin staining of normal and DDC-4-overexpressing mammary gland sections. Noticeably abnormal were cyst-like structures present occasionally in DDC-4/sFRP-4 transgenic glands, but generally, little evidence of abnormality was apparent. Infiltration by macrophages and eosinophils in the early lactating mammary glands was not elevated.

In situ hybridization using a *DDC-4/sFRP-4*-specific probe revealed that in early lactation (12 h after parturition), transgene expression was restricted to the epithelial cells, while fat cells and blood vessels were uniformly negative. In the epithelium, however, transgene expression was not homogenous; strongly positive areas were found beside negative ones (Figure 5d–f). Immunohistochemistry with affinity purified DDC-4-specific antibodies showed protein expression in agreement with the *in situ* hybridization results (Figure 5g and h).

We used the TUNEL assay to investigate *in situ* apoptotic cell death. In contrast to normal control tissues (Figure 6a and b) that showed very few apoptotic cells, DNA fragmentation in cells of the alveolar luminal epithelium were detected in large numbers in the alveoli of transgenic animals at late pregnancy (Figure 6c) and 12 h postpartum (Figure 6d). Cell death was also evident by the appearance of apoptotic bodies judged on the basis of nuclear size and shape (Figure 6c). A yellow-fluorescing artefact exhibited by erythrocytes, hemosiderin, and accumulations of debris must be ignored.

The DDC-4/sFRP-4-positive mammary glands continued to secrete milk, sufficient to nourish some of the young. Thus, it seemed likely that dying cells were being replaced with newly differentiated cells. In order to address this question, we have investigated cell proliferation using BrdU labeling during a 3-h *in vivo* treatment prior to killing. Indeed, an elevated proportion of BrdU-positive cells was observed in sections of line 718 mammary glands at day 2 of lactation (Figure 6f) as compared to the normal control (Figure 6e).

Caspases are a family of proteases whose activation is thought to represent an irreversible event in the apoptotic process. Caspase-3 has been shown to be ultimately involved in mammary epithelial involution.²¹ In order to obtain some insight into the relative expression of DDC-4/sFRP-4 and activated, cleaved caspase-3 as a marker for ongoing apoptosis, serial sections of mammary gland, line 718, taken 12 h after parturition, were stained. Serial sections compared staining with anti-cleaved caspase-3 antibody *versus* that with anti-DDC-4/sFRP-4 antibody. Along the alveolar and ductal lining of the lumen, only a small fraction of the epithelial cells exhibited moderately strong cleaved caspase-3 staining, although a much larger fraction were already DDC-4/sFRP-4 positive. Thus, either the cleaved-caspase-3 expressing fraction has a shorter half-life in the tissue, or the majority of

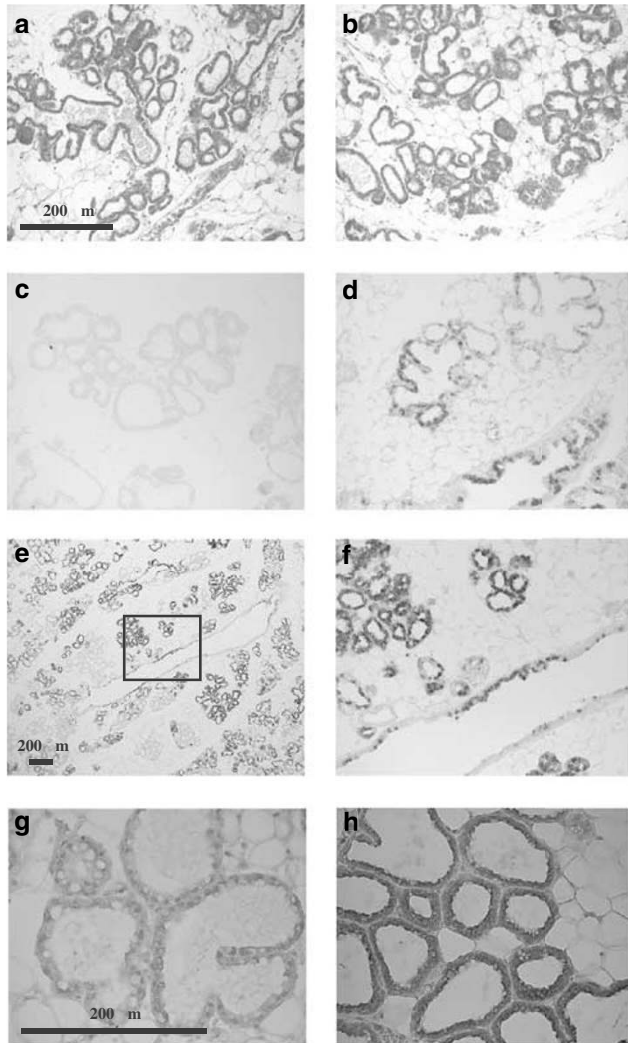


Figure 5 Histological appearance of DDC-4/sFRP-4 transgenic mammary glands. Hematoxylin and eosin staining of mammary glands 12 h postpartum from normal (a) and line 718 mice (b). (c–f) *In situ* hybridization with a DDC-4/sFRP-4 probes (c, sense and d–f, antisense) on transgenic mammary glands 12 h after parturition. The immunohistochemical detection of DDC-4/sFRP-4 in control C57BL/6 mice (g) and transgenic mice at 2 days of lactation (h) is shown. Note that essentially all luminal epithelial cells express DDC-4/sFRP-4 at this time. Magnifications are indicated by the bars in a (for a–d, f), in e at low magnification (expanded in f) and in g (for g and h)

the DDC-4/sFRP-4-positive cells fail to develop further to frank apoptosis. The residual milk secretion in the gland, allowing survival of half the progeny, speaks for the latter.

Caspase-3 enzymatic activity in mammary tissues from normal and transgenic mice

As demonstrated above, transgenic mammary glands show evidence of apoptosis in a time window between the last days of pregnancy and day 2 of lactation. A fluorescence assay for caspase-3 activity was performed *in vitro* to confirm the results

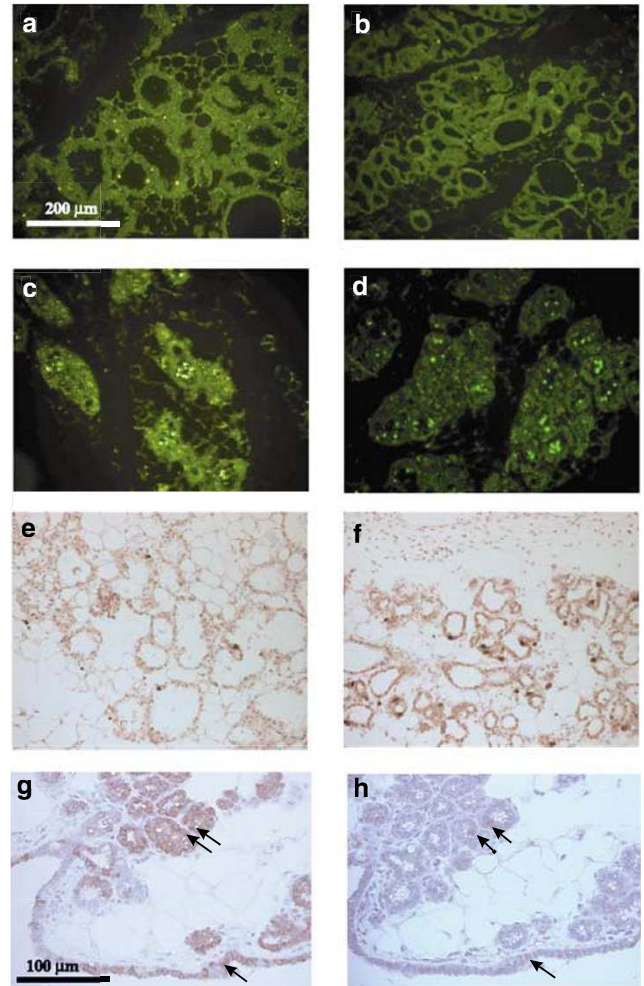


Figure 6 TUNEL reactions, used to document programmed cell death, are shown in (a)–(d). The TUNEL reaction was performed with fluorescent BODIPY-dUTP substrate on mammary gland sections from 20-day pregnant mice ((a) and (c)) and with mice 12 h after parturition ((b) and (d)). Mammary glands of C57BL/6 control mice ((a) and (b)) and transgenic line 718 mice ((c) and (d)). An *in vivo* BrdU labeling assay for cell proliferation was performed 3 h before killing. Immunohistochemical detection of incorporated BrdU is shown for normal C57/bl6 in (e) and in (f) for line 718 transgenic mammary glands at 2 days of lactation. Serial sections of mammary gland, line 718, taken at 12 h after parturition, are shown for comparison after staining with anti-DDC-4/sFRP-4 (g) or with anti-activated (cleaved) caspase-3 (Cell Signaling Technologies) (h) affinity-purified antibodies. The arrows indicate cells in the same locations. Such comparisons provide suggestive evidence that only a minority of the luminal epithelial cells exhibit a strong cleaved-caspase-3 positive staining, although many alveolar cells are already DDC-4/sFRP-4 positive. Magnifications are indicated by the bars in a (for a–f) and in g (for g and h)

obtained from the TUNEL reaction, the cleaved caspase-3 immunohistochemistry and the Northern blots for *SGP-2* expression, which together had indicated an onset of apoptosis congruent with transgene expression. The control animals were negative for activated caspase-3 at lactation and as expected exhibited caspase-3 activation already 1 day after forced weaning (Figure 7). In contrast, a three-fold induction of activated caspase-3 could be detected in mammary glands of line 718 transgenic animals already at day 19 of pregnancy. The peak caspase-3 activity

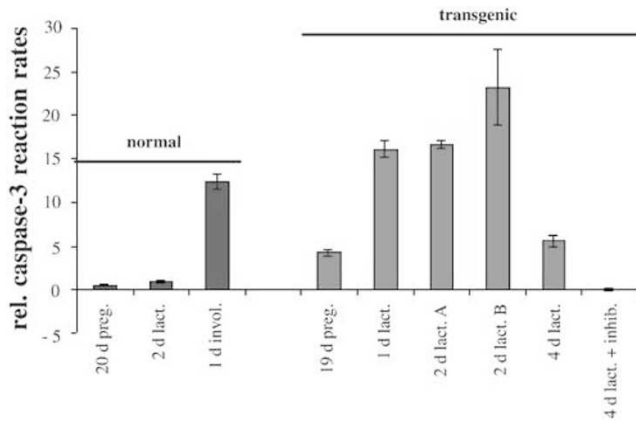


Figure 7 Schematic representation of caspase-3 activities as measured with the Z-DEVD-amino-4-methyl coumarin (AMC) substrate in a fluorescence assay. Normal C57BL/6 control mice (normal) were compared with line 718 transgenic animals at different days (d) of pregnancy (preg.), lactation (lact.), or involution (invol.). For the transgenic mammary glands at 2-day lactation, two separate animals (A and B) were studied. A reaction was performed with and without the reversible aldehyde inhibitor Ac-DEVD-CHO specific for caspase-3, in order to confirm specificity (4d lact, 4d lact + inhib.). Bars represent mean values; standard deviations are indicated

was detected in transgenic mammary glands at 1–2 days of lactation and declined thereafter (Figure 7). Thus, the appearance of apoptotic cells detected by the TUNEL assay is paralleled by the activation of caspase-3, indicating that unscheduled expression of DDC-4/sFRP-4 induces programmed cell death in the mammary epithelium.

Decline in PKB/Akt phosphorylation precedes onset of apoptosis and parallels loss of phosphorylation in GSK-3 β , BAD, and Forkhead

The question remains why the upregulation of DDC-4/sFRP-4 leads to apoptosis. From its predicted function as a secreted Frizzled competitor, DDC-4/sFRP-4 can be anticipated to block Wnt-Frizzled signaling, in this case probably by competing for available Wnt-4. From the work of several groups, one can speculate that Wnt-4 is the probable target for DDC-4/sFRP-4,^{17,22,23} but the link from Wnt signaling to a survival pathway remains somewhat elusive. Thus, we performed the experiments shown in Figure 8, which presents the results of a PKB/Akt protein kinase assay and of Western blot experiments, together documenting a major survival pathway, the PI-3 kinase (PI3 K), and PKB/Akt pathway, through which many growth factors maintain cell survival. The study was performed within a narrow time window in which mammary glands contain a maximal proportion of luminal epithelial cells (from 19 days pregnancy to 2 days of involution). Samples were normalized to a constant amount of PKB/Akt protein as detected with Western blot, then parallel blots were probed with phosphorylation-specific, affinity-purified antibodies for PKB/Akt itself, for GSK-3 β , for the proapoptotic BAD, which is held in the inactive form through phosphorylation, and for the transcription factor Forkhead. The results in Figure 8 indicate that PKB/Akt kinase activity is greatly reduced in line 718 samples, taken both in pregnancy

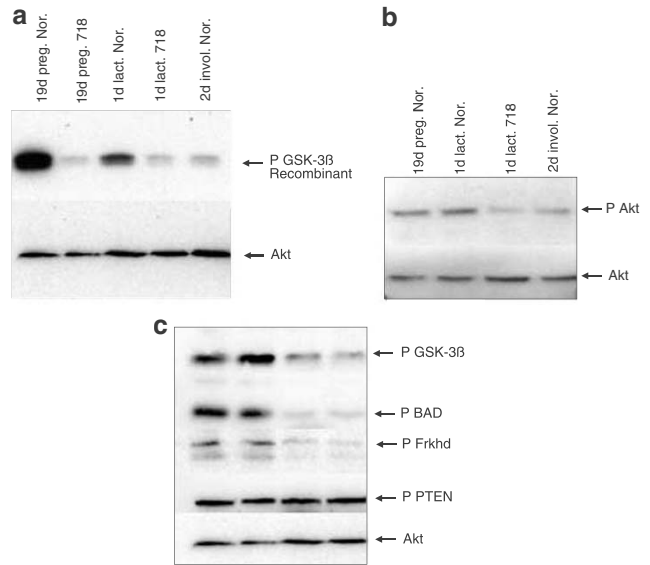


Figure 8 (a) PKB/Akt kinase activity was measured on tissue samples of mammary glands obtained from normal C57BL/6 mice at 19 days of pregnancy (19d preg. Nor.), lactation 1 day (1d lact. Nor) and 2 days of involution after forced weaning (2d invol. Nor.) and from line 718 mice at 19 days pregnancy (19d preg. 718) and 1 day of lactation (1d lact. 718). Tissue samples were lysed (see Materials and methods), immunoprecipitated with immobilized anti-PKB/Akt antibody and subjected to a kinase reaction in the presence of recombinant GSK-3 β and ATP. PAGE and electroblotting were performed and Western blots analyzed with antibodies specific for phospho-GSK-3 β and PKB/Akt. The latter was a control for equal loading. (b) The phosphorylation of PKB/Akt was analyzed by PAGE followed by immunoblotting of several mammary gland tissue samples from normal C57BL/6 and line 718 transgenic mice. These samples had been rapidly lysed in sodium dodecyl sulfate-containing sample buffer. Specific antibody reactive with phospho PKB/Akt (Ser-473) demonstrated the activated fraction, while affinity-purified anti-PKB/Akt antibody applied to a parallel blot indicated the equivalence of loading. (c) Several substrates of PKB/Akt were lysed, separated on PAGE and analyzed in Western blots utilizing phospho-specific, affinity-purified from Cell Signaling Technologies. Arrows indicate phospho-GSK-3 β , phospho-BAD (Ser-136), phospho forkhead (Ser-256), phospho PTEN (Ser-380) as well as the control for equivalence of loading, anti-PKB/Akt

and in lactation. Likewise, PKB/Akt phosphorylation is reduced in control normal tissues at 2 days of involution and in the line 718 transgenic 1 day lactation sample. With the onset of apoptosis in the involuting mammary glands of the normal control mice, GSK-3 β phosphorylation, and most significantly BAD and Forkhead phosphorylation is decreased. Since the phosphorylated form of BAD is known to be inactive, the release of the proapoptotic form seems likely to have an impact in the initiation of apoptosis. While the normalization was to the amount of total PKB/Akt detected in the blots, it is interesting that PTEN phosphorylation, an activity upstream of the PKB/Akt, impacting mainly on PI3 K itself, exhibited essentially no effect from either the DDC-4/sFRP-4 over-expression in line 718, or from the onset of involution in the normal controls.

Discussion

The defining signals responsible for the transition from lactation to involution in the mammary gland are still unknown.

While procedures that induce a local involution in sealed mammary glands have shown that regulation by systemic hormones cannot be the main determining factor in the onset of involution,²⁴ there remain several other hypotheses. For example, transforming growth factor β 3 has been proposed.²⁵ Most hypotheses, however, focus on the loss of survival signals. Extracellular matrix–integrin interactions presumably represent an important link between adhesion and the events of apoptosis.²⁶ In this context, one can speculate that the induction of tissue remodeling proteases capable of degrading basement membrane might be an early trigger for programmed cell death.²⁷ Recent work, however, tends to show that the earliest events of apoptosis occur before detectable changes in extracellular matrix or cognate integrin receptors can be detected.²⁸ Other survival signals of importance are the insulin-like growth factors (IGFs), whose function can be blocked by IGF-binding proteins (IGFBP). For example, IGFBP-5 is produced very early at the onset of involution; hence, it could well be a relevant mediator of apoptosis in mammary involution.²⁹ However, for every mechanism thus far proposed, just as for the role of DDC-4/sFRP-4 described above, it remains to question as to what sensing system triggers the involution program. Quite possibly, engorgement and physical strain communicate the end of lactation and beginning of involution, presumably via changes in gene expression.³⁰

The importance of the Wnt/Frizzled signaling pathway in mammary development has emerged only very gradually.³¹ *Wnt-1* was early recognized as the oncogene responsible for many of the MMTV-induced neoplasms. *Wnt-1* was overexpressed inappropriately in the mammary gland as a result of a downstream promotion caused by the nearly random integration of the MMTV. Since *Wnt-1* is not normally expressed in the mammary gland at any developmental stage and since it can apparently interfere with the action of some other *Wnts* on its receptor, a potential for dysregulation is realized, the consequence being a mammary tumor.

DDC-4 belongs to the sFRP family, recently recognized as regulators of the Wnt signaling system.^{12,13,32} Their role as extracellular competitors for Wnt ligands is to reduce the probability that Frizzled receptors are activated, that is, to block the Wnt/Frizzled signaling pathway. Although both the ligand with which DDC-4/sFRP-4 binds and the Frizzled receptor that it blocks are still unknown, it was to be anticipated that like the expression of *Wnt-1*, inappropriate DDC-4/sFRP-4 expression would probably disturb Wnt/Frizzled regulation in the tissue. We hypothesized that a necessary survival signal may be blocked, leading to the apoptotic pathway.

The approaches employed here to show the phenotypic consequences of ectopic DDC-4/sFRP-4 expression have not previously been used in combination. The transgenic mammary gland approach traditionally relies on epithelial reconstitution to demonstrate a phenotypic effect.^{17,22} The effects reported in the past have been hyperplasia and tumor induction, and apparently did not suffer from the declining expression that is probably unavoidable with this approach. Our experiments differ and illustrate that transplantation of cells secreting a soluble effector can generate a local phenotype that is readily observable acting on the endogenous, normal epithelium. Unfortunately, our experience was

that *ex vivo* infected cells, even primary cells, which should exhibit a normal phenotype, take on characteristics during their brief stay in culture inducing a reactive tissue response. This effect is quite apparent in a system that lacks any additional proliferative stimulus. With our second approach to study DDC-4/sFRP-4 overexpression, the transgenic mouse model, we could avoid such immune reactions.

Transgenic animals have been established, which overexpress a variety of oncogenic, growth-inducing^{15,33} or antiapoptotic³⁴ genes. Unusual in our study is the overexpression of a presumptively proapoptotic gene product, DDC-4/sFRP-4. Such an experiment suffers from several problems, most notably the risk that an apoptotic phenotype may not be sufficiently synchronous to allow dying cells to be observed before their removal by the action of phagocytes. In fact, the DDC-4/sFRP-4-overexpressing transgenic mice illustrate this problem in early lactation. It is noticeable, that while maximal apoptosis measured by TUNEL reaction is observed within 12 h after parturition, the peak activity of caspase-3 was detected somewhat later and remained elevated longer.

The significance of the observed apoptotic potential of DDC-4/sFRP-4 lies in the congruence between the observations made on its expression in normal physiological apoptosis in several tissues and in mammary glands of transgenic DDC-4/sFRP-4 overexpressors. In essence, these experiments serve as a proof of principle. Where previously an association of DDC-4/sFRP-4 upregulation with apoptosis was recognized, we can now speak of causation, at least in a limited sense.

A probable mechanism by which DDC-4/sFRP-4 causes cell death is by abrogating PI3 K-PKB/Akt survival signaling: PKB/Akt binds to PI3 K-generated phospholipids and translocates from the cytoplasm to the inner surface of the plasma membrane, where PI3 K-generated 3'-phosphorylated phospholipids reside. Relocalization to the plasma membrane brings PKB/Akt in proximity to regulatory kinases that phosphorylate and activate PKB/Akt.³⁵ Four sites (Ser-124, Thr-308, Thr-450, and Ser-473) were identified on Akt1 that can be phosphorylated *in vivo*.³⁶ However, PKB/Akt activity appears to depend on phosphorylation of Thr-308 and Ser-473; Ser-124 and Thr-473 seem to be basally phosphorylated.³⁵ Thr-308, in the presence of PI3,4,5P, becomes phosphorylated by 3-phosphoinositide-dependent protein kinases (PDKs).^{35,37,38} Ser-473 may become phosphorylated through a complex consisting of PDK-1 and PRK-2 (protein kinase C-related kinase-2), and by integrin-linked kinase (ILK), a serine/threonine kinase that interacts with the cytoplasmic tail of integrin β subunits.³⁵ Activation of PKB/Akt results in the phosphorylation of, among others, glycogen synthase kinase 3 (GSK3), the proapoptotic Bcl-2 family member BAD, caspase-9, and Forkhead transcription factors.^{35,39,40} Note that, although BAD functions as a proapoptotic molecule, its phospho-form represents an inactive state not inducing cell death.³⁹

Interestingly, inactivation of GSK-3 β through phosphorylation may result in nuclear accumulation of β -catenin⁴⁰ and thereby provides a link, at least in one direction, to the classical Wnt/ β -catenin pathway. Our data presented here support this view. Being a member of the sFRP family, it is

probable that DDC-4/sFRP-4 abrogates a Wnt-regulated signal transduction pathway. Since involuting normal mammary glands as well as DDC-4/sFRP-4-overexpressing transgenic glands at lactation showed reduced levels of both PKB/Akt kinase activity, and reduced GSK-3 β , BAD and Forkhead phosphorylation (Figure 8), we conclude that the observed correlation between DDC-4/sFRP-4 expression and increased rates of apoptosis is because of an inactivation of a PKB/Akt-dependent survival pathway.

Although our observations can best be explained by functional links between Wnt signaling and the PI3 K-PKB/Akt pathway, we do not know at which biochemical level the crosstalk between the two signaling systems occurs. GSK-3 β may be the point of convergence. GSK-3 β is recognized as an important modulator of apoptosis. GSK-3 β , for instance, downregulates the activities of several transcription factors that are critical promoters of cell survival, such as heat shock factor-1 (HSF-1) and cyclic AMP response element binding protein (CREB).⁴¹ Furthermore, caspase-3 activation induced by inhibition of mitochondrial complex I has been shown to be facilitated by GSK-3 β .⁴² Since both PI3K-PKB/Akt as well as Wnt signaling inhibit GSK-3 β activity, one might speculate that a DDC-4/sFRP-4-induced inhibition of the Wnt pathway reactivates GSK-3 β and thereby promotes cell death. Recent reports by Song *et al.*⁴³ and Fukumoto *et al.*⁴⁴ indicate that the Wnt pathway can phosphorylate and activate PK-B/Akt via another intermediate player, dishevelled. Their results differ in the involvement of GSK-3 β in this crosstalk. Significant in the context of our results, however, is that Fukumoto *et al.*⁴⁴ show not only that downregulation of the PI3 K and PKB/Akt pathway parallels a reduced GSK-3 β phosphorylation, thus inhibiting Wnt signaling, but also, most interestingly, that Wnt signaling through dishevelled is able to activate PKB/Akt. Thus, the crosstalk is not just one way, it is 'hand-shaking'.

In summary, we have described the role of DDC-4/sFRP-4 in inducing apoptosis. Late pregnant and lactating mammary glands of DDC-4/sFRP-4-overexpressing transgenic mice showed pronounced cell death as determined by Northern blot analysis for sulfated glycoprotein-2, immunohistochemistry for cleaved caspase-3, as well as in TUNEL and caspase-3 assays. We have previously shown that DDC-4/sFRP-4 was upregulated in various tissues undergoing physiological apoptosis.^{3,4,45} The new data show that DDC-4/sFRP-4 expression is a stimulus rather than a consequence of the apoptotic program in the mammary gland. Since DDC-4 is an sFRP and thus thought to sequester Wnt ligands, we propose a role for DDC-4/sFRP-4 in abrogating a Wnt-regulated survival pathway, the latter possibly signaling via PKB/Akt. Since PI3 K and PKB/Akt appears to delay the onset of p53-mediated, transcriptionally dependent apoptosis,⁴⁶ it would be interesting to explore the potential of DDC-4/sFRP-4 in overriding the p53-associated cell death inhibition mechanism active in many human tumors.

Materials and Methods

Construction of the DDC-4/sFRP-4 retroviral vector

The DDC-4/sFRP-4 cDNA, containing the entire coding sequence, was employed as template for the retroviral construct.⁴ Because of the

absence of suitable restriction enzyme sites that would allow the coding information to be cloned into the pBabe^{puro} vector without extensive 5' and 3' untranslated regions, we used PCR to obtain a subfragment spanning the coding sequence associated with short (20–30 bp) 5' and 3' untranslated regions. The primers were constructed to contain restriction enzyme sites near their 5' termini, *Bam*H1 for the sense, and *Eco*RI for the antisense primers. The resulting PCR product was restricted, purified and cloned into the respective sites of the pBABE^{puro} murine leukemia virus vector.⁴⁷ Preparation of packaged virus was performed in BOSC cells derived from the human 293 kidney tumor line.¹⁶

Culture of clone 31D mouse mammary epithelial cells, clone 30F mammary fibroblastoid cells and primary mammary fibroblasts

Epithelial 31D and fibroblast-like 30F mammary cell lines, capable of differentiation *in vitro*, were derived from BALB/c mid-pregnant mammary glands in our laboratory.^{48,49} Monolayer cultures maintained in medium with 10% fetal calf serum supplemented with insulin (5 μ g/ml) were infected with the pBabe^{puro} retroviral constructs, with and without the DDC-4/sFRP-4 insert. Selection with puromycin at 25 μ g/ml was begun after 24 h and maintained for at least 96 h. Cultures were propagated for up to 10 weeks, with reselection at intervals. Samples for RNA isolation were taken weekly.

Primary mammary fibroblast cells were prepared from mid-pregnant BALB/c mice. Mammary glands were minced on dry cell culture plates to fragments <1 mm³. The fragments were transferred to new culture dishes and allowed to dry for 5 min at room temperature after which Eagle's Dulbecco-modified medium (EDM) containing 10% fetal calf serum was added. After 4–5 days, the dish was confluent with fibroblasts growing out of the fragments. The primary fibroblast cultures were trypsinized briefly, trypsin removed and the cells resuspended in EDM containing 10% fetal calf serum. The fragments were allowed to settle out, and the suspension of single cells was centrifuged at 1000 \times g for 3 min. The pellet was resuspended in EDM with 1% fetal calf serum, polybrene (final concentration 8 μ g/ml) and the virus stock suspension (multiplicity of infection approximately 0.5). Cultures were exposed for 60 min at room temperature to the virus. Thereafter, the suspension was again pelleted, and finally the cells plated in 5% fetal calf serum containing EDM. After 24 h, puromycin (final concentration 25 μ g/ml) selection was begun. After selection for 4 days, puromycin was removed. An approximately three-fold expansion of the fibroblast culture was achieved by 7 days.

Transplantation of *ex vivo*-infected cells into BALB/c mice

Cultures of cells were trypsinized, resuspended in EDM containing 10% fetal calf serum briefly to inhibit the protease and then pelleted twice successively in cold EDM without serum. Dense suspensions of approximately 4–6 \times 10⁶ cells/ml were prepared and 20–40 μ l amounts transplanted into pockets made in the mammary fat pads of the fourth glands prepared with fine forceps just medial from the major, large mammary lymph node. BALB/c mice were either 5-week-old pubertal females, or mature females at the 17 days of pregnancy. Mice were anesthetized using pentobarbitol, and the wound was sutured carefully to close tightly. For studies of puberty, mice were killed after 1 week; transplantation into pregnant mice was analyzed 6 days after implantation, in the lactating state 2 days after delivery of normal litters. Mammary glands were examined as whole mounts, or each gland was divided into

fragments ca. 1.5 cm long to provide material for both RNA isolation (frozen at -80°C in lysis buffer) and fixation in freshly prepared 4% formaldehyde solution at 4°C for 16 h.

Preparation of rabbit polyclonal, affinity-purified DDC-4/sFRP-4 antibodies

The *DDC-4/sFRP-4* coding sequence was cloned in frame into the appropriate pGEX expression plasmid and the glutathione *S*-transferase-DDC-4 fusion protein expressed in *Escherichia coli*. The fusion protein was isolated by preparative SDS-PAGE. Rabbits received multiple, intradermal injections of approximately 500 μg fusion protein, initially with Freund's complete and thereafter with Freund's incomplete adjuvant. Antibodies were affinity purified by absorption to antigen coupled to Reacti-Gel HW-65F support (Pierce, Rockford, IL, USA). Specific antibody was eluted using 100 mM glycine buffer, pH 2.5 and dialyzed against PBS.

PKB/Akt protein kinase assay, SDS-PAGE, immunoblotting, and antibodies

Tissue lysates were prepared from rapidly harvested, ice-cold samples lysed with the help of a Polytron in a buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β -glycerophosphate, 1 mM sodium orthovanadate, 1 $\mu\text{g}/\text{ml}$ leupeptin, and immediately before lysis, 1 mM PMSF.

The PKB/Akt protein kinase assay employed a kit supplied by Cell Signaling Technology (Beverly, MA, USA). The principle is an immune precipitation using immobilized anti-Akt antibody to recover the enzyme quantitatively, followed by a reaction at 30°C in the presence of a recombinant fusion protein with GSK-3 peptide substrate and ATP. The phosphorylated product is detected in a Western blot using phospho-GSK-3-specific antibody.

Conventional vertical SDS-PAGE was performed with 11% acrylamide-bis-acrylamide according to the method of Laemmli.⁵⁰ Immunoblotting onto nitrocellulose filters was accomplished with a semidry transfer procedure, followed by blocking of the membrane with 4% nonfat dried milk for 60 min in Tris-buffered saline (TBS: 25 mM Tris, pH 7.5, 150 mM sodium chloride).

The rabbit polyclonal anti-DDC-4 antiserum was employed at a dilution of 1:1000. Anti-Akt, anti-phospho-ser473 Akt, anti-phospho-ser9 glycogen synthetase kinase-3 β and anti-phospho-ser136 BAD, anti-phospho-ser256 Forkhead and anti-phospho-ser380 PTEN were rabbit antipeptide antibodies obtained from Cell Signaling Technology (Beverly, MA, USA). All were used at a final dilution of 1:1000 except anti-phospho-ser136 BAD that was employed at 1:500 dilution. Detection was performed with an anti-rabbit IgG antibody conjugated with horse radish peroxidase at 1:2000 dilution (Cell Signaling Technology, Beverly, MA) according to the procedures recommended by the supplier. Luminol was employed as the substrate and the chemoluminescent signal was acquired with a Roche Diagnostics (Rotkreuz, Switzerland) Lumi-Imager.

Preparation of MMTV-LTR-DDC-4 construct and establishment of transgenic animals

The complete coding sequence of the *DDC-4/sFRP-4* cDNA was purified, taking advantage of convenient Kpn-I restriction sites. Ligating on *NotI* adapters (Roche Diagnostics, Rotkreuz, Switzerland) allowed the construction of an MMTV-LTR expression cassette⁵¹ containing a 1.4 kb cDNA fragment covering the 1.05 kb coding sequence of *DDC-4/sFRP-4*.

SalI digestion was employed to obtain the entire cassette for microinjection into pronuclei of zygotes generated from F1 hybrid female mice (C57BL/6 \times DBA/2) mated to DBA/2 males. Pronuclear injection was prepared at the Animal Facility of the Center for Molecular Biology (University of Heidelberg, Germany). From the resulting offspring, DNA was prepared from tail biopsies and positive founder mice identified by PCR. Eight founder lines were obtained, of which two expressed the transgene in the mammary glands of pregnant or lactating animals.

Isolation of RNA and Northern blotting

Total RNA was prepared from tissue samples using the guanidinium-thiocyanate extraction method of Chomczynski and Sacchi.⁵² In total, 5 μg of total RNA was denatured with glyoxal, electrophoresed on a 1% agarose gel and blotted to nitrocellulose. Equal loading was controlled by examination of ribosomal RNA bands following staining with acridine orange as previously described.³ Probes for hybridization were prepared using the Random Primed Labeling Kit (Roche Diagnostics, Rotkreuz, Switzerland) including ³²P-dCTP (800 Ci/mM; Amersham International, Little Chalfont, England) in the reaction and hybridization was carried out as previously described.³

In situ hybridization, immunohistochemical detection of DDC-4/sFRP-4, incorporated BrdU and activated (cleaved) caspase-3

Fixation, *in situ* hybridization and immunohistochemical detection was carried out as previously described.^{45,53} The rabbit affinity purified anti-DDC-4 serum was used at a dilution of 1:100 on tissue sections after microwave treatment in 50 mM sodium citrate for 5 min at reduced energy (maximum temperature, 90°C). For BrdU labeling, females were injected I.P. with 0.2 ml of BrdU (50 mg/ml) in PBS 3 h prior to killing. Anti-BrdU antibody was obtained from Roche Diagnostics. For the detection of activated (cleaved) caspase-3, an antibody specific for the cleaved caspase-3 (asp175) was obtained from Cell Signaling Technology.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL)

The TUNEL reaction was performed as previously described⁴⁵ using either BODIPY-dUTP visualized directly in the fluorescence microscope (Molecular Probes, Eugene, OR, USA) or digoxigenin-11 dUTP detected with anti-digoxigenin *Fab* antibodies conjugated to alkaline phosphatase (Roche Diagnostics, Rotkreuz, Switzerland). Fixation was exactly as for *in situ* hybridization. For the TUNEL, optimization of proteinase K treatment proved very critical and was carried out with 5 $\mu\text{g}/\text{ml}$ proteinase K in Tris 20 mM, pH 7.8, at 30°C for 15 min.

Fluorescence assay for caspase-3 activity

Numerous small tissue samples (50 mg) were obtained from each mammary gland and frozen at -80°C . Lysates were prepared in 1% Triton X-100 (PBS) and were sonicated 2×30 s on ice with a 5 mm probe sonifier set to 50 W. Following centrifugation at 4°C and $50\,000 \times g$, 100 μg amounts of protein were reacted with Z-DEVD-amino-4-methyl coumarin (AMC) substrate according to the manufacturer's instructions (Molecular Probes, Eugene, OR, USA). Reactions were performed in duplicate, with and without the specific inhibitor Ac-DEVD-CHO.

Fluorescence at 460 nm was measured over 30 min at 30°C in a fluorometer with excitation at 380 nm.

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