Mouse Mammary Tumor Virus p75 and p110 CUX1 Transgenic Mice Develop Mammary Tumors of Various Histologic Types

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Abstract
The p75 and p110 isoforms of the CUX1 homeodomain protein are overexpressed in breast tumors and cancer cell lines. To assess and compare the ability of these short CUX1 isoforms in driving mammary tumor development, we used site-specific transgenesis into the Hprt locus to generate transgenic mice expressing p75 or p110 CUX1 under the control of the mouse mammary tumor virus-long terminal repeat. We report that mammary tumors developed after a long latency period, and although various histopathologies were observed, the proportion of adenosquamous carcinomas was significantly higher in p75 CUX1 than in p110 CUX1 transgenic mice. Metastasis to the lung was observed in three p75 CUX1 transgenic mice. Comparisons between tumors and adjacent normal mammary glands revealed that transgenes were overexpressed in most but not all tumors, yet in all cases tested, CUX1 DNA binding was increased, suggesting that both higher expression and changes in post-translational modifications can contribute to stimulate transgene activity. Interestingly, higher expression of erbB2 mRNA was seen in most tumors, not only solid carcinomas but also adenosquamous carcinomas, whereas higher expression of various Wnt genes and activation of the β-catenin pathway was observed primarily in adenosquamous carcinomas. Activation of erbB2 expression appeared to represent a cooperating event that occurred independently of CUX1. In contrast, chromatin immunoprecipitation, short hairpin RNA–mediated knockdown, and reporter assays established that CUX1 is involved in the transcriptional regulation of several Wnt genes. Together, these results support the notion that oncogenic activity of CUX1 can facilitate the establishment of a Wnt/β-catenin autocrine loop. [Cancer Res 2009;69(18):7188–97]

Introduction
CUX1 is the mammalian orthologue of the Drosophila Cut homeodomain protein that has previously been called CCAAT displacement protein or cut homeobox (Cux; reviewed in refs. 1, 2). In contrast to the full-length p200 CUX1 protein, which binds rapidly but only transiently to DNA, both p75 and p110 CUX1 bind stably to DNA and function as transcriptional repressors or activators depending on the promoter (3–7).

From cell-based assays, CUX1 plays a role in at least two distinct processes: cell motility and invasion and cell cycle progression. Small interfering RNA–mediated knockdown of CUX1 expression caused a decrease in cell motility and invasion and in pulmonary colonization after caudal vein injection (8). In several cell types, populations of cells stably expressing p110 CUX1 reached the S phase faster than control cells (9). Similar effects have been observed with the p75 isoform.5

Accumulating evidence suggests that CUX1 may function as an oncogene. Cux1 inactivation hindered both the formation of foci on a monolayer and tumor growth in mice (9). Elevated CUX1 mRNA and protein expression was reported in primary tumors and cancer cell lines of various types (8, 10–12). In situ hybridization performed on multiple tissue core arrays showed increased CUX1 expression within high-grade, but not low-grade, breast carcinomas (8). Among patients with grade 3 breast tumors, CUX1 mRNA expression inversely correlated with relapse-free and overall survival (8). We have shown that the proteolytic processing of CUX1 is tightly regulated during the cell cycle in normal cells but becomes constitutive in many transformed cells (5, 13). Among invasive breast tumors, a significant association was established between higher intron 20-mRNA and p75 CUX1 expression and a diffuse infiltrative growth pattern (6). Transgenic mice expressing p200 CUX1 under the control of the cytomegalovirus enhancer/promoter displayed multiorgan hyperplasia and organomegaly (14). Hyperplasia in the liver was associated with various lesions including the development of mixed cell foci and hepatocellular carcinomas. Unfortunately, the cohorts of mice were too small to be able to reach a firm conclusion as to the implication of CUX1 in this type of malignancy (15).

A causal role for CUX1 in breast cancer has not yet been shown. Moreover, whether different isoforms of CUX1 impart distinct phenotypes remains to be addressed. To compare the effect of overexpressing the p75 and p110 CUX1 isoforms in mammary epithelial cells, we have constructed mouse models using site-specific transgenesis (16). In mixed genetic backgrounds, many virgin p75 CUX1 mice succumbed to myeloproliferative disease–like myeloid leukemias (17). In the present study, we report that, in the FVB genetic background, multiparous transgenic mice carrying a p75 or p110 CUX1 transgene developed late-onset mammary tumors of various histologic subtypes. These results provide the first in vivo evidence for a causal role of CUX1 in the pathogenesis of breast cancer.

Materials and Methods

Generation of transgenic mice. The p75-CUX1 and p110-CUX1 transgenic mice were generated by site-specific transgenesis into the Hprt locus as described in ref. 17. Each line was backcrossed for at least seven generations with mice of the FVB strain. Two lines of p75 CUX1 transgenic mice were generated; as expected, transgene expression in the FVB genetic

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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5 Sansregret and Nepveu, unpublished observations.
Figure 1. Expression of CUX1 transgenes in the mammary gland during development. A, p75 and p110 CUX1 coding sequences, under the control of the MMTV-long terminal repeat, were introduced by specific transgenesis into the Hprt locus. The epitopes recognized by the 861, 1062, and 1300 CUX1 antibodies are shown. B, expression of p75 and p110 CUX1 transgenes in the mammary gland was analyzed by reverse transcription-PCR at 5 wk (virgin; 5w), 3 mo (virgin; 3m), 7.5-d pregnancy (P), 6-d lactation (6L), and 4-d involution (4I). Primers used are shown by arrows in A. C, immunohistochemical staining of mammary glands in 5-week-old virgin, 3-month-old virgin, and 7.5-d pregnant mice. Results are shown for wild-type, p75, and p110 CUX1 mice. D, whole-mount analysis of mammary gland development in virgin mice at ages 5 wk and 3 mo (left). Quantification of the enhanced ductal outgrowth observed in p75 CUX1 mice at age 5 wk (right). Five mice per line were analyzed at each time point. *, P < 0.05 (Student's t test).
background was found to be identical in the two lines. To study tumor burden, we generated cohorts of female mice carrying one copy of the transgene on one chromosome X. As a result of random inactivation of one X chromosome in each cell, the transgene would be expected to be expressed in ~50% of cells in females.

Reverse transcription-PCR analysis. RNA purification and reverse transcription-PCR were done as described previously (17).

Whole mounts. In situ mammary gland number 4 was spread on a glass slide, air-dried, and fixed overnight in acetone. The tissue was stained in Harris hematoxylin, dehydrated in 70% ethanol followed by 100% ethanol and xylenes, and then mounted in Permount.

Immunoblotting. Total protein extracts were prepared as described (18). Western blot analyses with anti-CUX1 1300 (1:1,000), anti-erbB2 (Calbiochem; OP15; 1:1,000), anti-cytokeratin 18 (Abcam; Ab7797; 1:1,000), anti-β-tubulin (Sigma; T6557; 1:1,000), or anti-actin (Santa Cruz Biotechnology; SC1616; 1:1,000) were done as described previously (5).

Histology and immunohistochemistry. Formalin-fixed organs were embedded in paraffin and cut in sections of 5 μm for H&E staining. Alternatively, immunohistochemistry was done as described (18). The following primary antibodies were used: anti-CUX1 1300 and 861 (1:500), cytokeratin 6 (Covance; PRB-169P; 1:250), cytokeratin 14 (Covance; PRB-155P; 1:250), cytokeratin 8/18 ( Fitzgerald Industrias; 20R-CP004; 1:100), or β-catenin (BD Biosciences; 610153; 1:500).

Chromatin immunoprecipitation. Chromatin immunoprecipitation was done with 4 × 10⁶ synchronized Hs578t cells as described previously (7, 18).

Cell culture and stable cell lines establishment. NMuMG<sup>NYPD</sup> cells were infected with pLXSN empty vector (Clontech Laboratories) or p75/CUX1HA-containing vector and selected with G418 to generate stable cell lines. Retrovirus production was done as described (9). For conditionally knockdown of CUX1 in Hs578t cells, we took advantage of the Addgene lines. Retrovirus production was done as described (9). For conditional CUX1HA-containing vector and selected with G418 to generate stable cell line.

Solid carcinoma 4 (29) 5 (56)

Adenomyoepithelioma 1 (11)

Adenoma: tubular acinar 1 (11)

Adenosquamous carcinoma 10 (71)* 2 (22)

NOTE: One p75 CUX1 mouse developed two distinct tumors: an adenosquamous carcinoma in one mammary gland and a solid carcinoma in another mammary gland.

* A significantly higher proportion of adenosquamous carcinomas were observed in p75 CUX1 mice (P = 0.0361).

Table 1. Distribution of histopathologic types in mammary tumors from p75 and p110 CUX1 transgenic mice

<table>
<thead>
<tr>
<th>Type of mammary tumors</th>
<th>p75 CUX1, n (%)</th>
<th>p110 CUX1, n (%)</th>
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<tbody>
<tr>
<td>Adenosquamous carcinoma</td>
<td>10 (71)*</td>
<td>2 (22)</td>
</tr>
<tr>
<td>Adenoma: tubular acinar</td>
<td></td>
<td>1 (11)</td>
</tr>
<tr>
<td>Adenomyoepithelioma</td>
<td></td>
<td>1 (11)</td>
</tr>
<tr>
<td>Solid carcinoma</td>
<td>4 (29)</td>
<td>5 (56)</td>
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Tail vein injections. One million NMuMG<sup>NYPD</sup> vector or p75 cells were injected into the tail vein of nude mice (25 mice for each cell line). Lungs were harvested 90 days after injections.

Electrophoretic mobility shift assay and South-Western. Electrophoretic mobility shift assay was done as in ref. 5. South-Western blotting was done as described previously using a double-stranded oligonucleotide probe containing the CUX1 consensus-binding site: CCGATATCCTGAT (20).

Luciferase assays. Luciferase assays were done as described (5). The reporter plasmids derived from the pGL3 basic vector were generated by inserting DNA sequences corresponding approximately to -1,000 to +100 relative to the transcription start site: Wnt1 from -1,297 to +98, Wnt6 from -1,254 to +220, Wnt8b from -2,314 to +77, and Wnt10A from -904 to +29.

Results

Generation of mouse mammary tumor virus-long terminal repeat p75 and p110 CUX1 transgenic mice. To assess and compare the oncogenic potential of p75 and p110 CUX1, we set out to generate transgenic mice that would express these isoforms specifically in mammary epithelial cells. The transgenes contained coding sequences for p75 or p110 CUX1 downstream of the mouse mammary tumor virus (MMTV)-long terminal repeat. To avoid complications resulting from variations in copy number and integration site effects, we used the method of targeted transgenesis to insert the construct into the mouse Hprt locus (16). After eight backcrosses into the FVB strain of mice, females were made multiparous. Transgene expression was monitored by reverse transcription-PCR and immunohistochemical staining and exhibited the pattern usually observed with MMTV-long terminal repeat–driven transgenes. Expression was elevated at age 5 weeks, returned to a low level at 3 months in virgin mice, and later increased during early pregnancy and lactation (Fig. 1B and C). Note that immunohistochemical staining with the CUX1 antibodies was not sensitive enough to detect expression of the endogenous CUX1 protein in wild-type mice (Fig. 1C, top). We conclude, therefore, that the staining observed in transgenic mice must reflect transgene expression (Fig. 1C). A confirmation of this notion was provided from the comparison of results obtained with the 861 and 1300 CUX1 antibodies. Whereas staining was observed in both p75 and p110 CUX1 transgenic mice with the 1300 CUX1 antibodies, as predicted,
staining was seen only in the latter mice with the 861 CUX1 antibodies that recognize an epitope present only in the p110 isoform (Fig. 1C, compare 7.5-day pregnant 1300 and 861 in p75 and p110 CUX1 mice). Note that, because of random X chromosome inactivation, at best 50% of cells were expected to express the transgene because female mice were heterozygous at the Hprt locus: one allele carried the transgene and one did not. In practice, however, transgene expression was observed in <50% of mammary epithelial cells (Fig. 1C; data not shown).

**Ductal outgrowth at age 5 weeks in p75 CUX1 transgenic mice.** Mammary gland whole mounts were prepared from wild-type, p75 CUX1, and p110 CUX1 transgenic mice at ages 5 weeks and 3 months. At 5 weeks, a faster ductal outgrowth was observed in the p75 CUX1 but not in the p110 CUX1 transgenic mice (Fig. 1D). At 3 months, no difference was noted between wild-type and transgenic mice, in agreement with the observed low transgene expression at that time (Fig. 1D).

**MMTV p75 and p110 CUX1 transgenic mice develop late-onset mammary carcinomas.** Cohorts of multiparous p75 CUX1 (n = 70), p110 CUX1 (n = 74), and wild-type FVB mice (n = 88) were monitored for tumor incidence over 2 years (Fig. 2A, Kaplan-Meier plots). Tumors were detected in many organs and tissues, and overall tumor incidence was 33% and 53% in p75 and p110 CUX1 transgenic lines, respectively, compared with 22% in wild-type FVB/N mice.

Figure 3. Some mammary tumors from p75 CUX1 transgenic mice metastasize to the lungs. A, H&E stainings of primary tumors and lung metastases. Reverse transcription-PCR analysis of β-casein and GAPDH mRNA in a normal mammary gland (MG) and in lung sections from mice with lung metastases (lung M) or with a primary lung tumor (lung P). B, populations of NMuMG\(^{\text{vprf}}\) mouse mammary epithelial cells stably expressing p75 CUX1 were submitted to two-chamber assays. Top cells were removed and the average pixel count was measured to evaluate the number of migrating cells. Representative results from at least three independent experiments are presented. **, \(P < 0.01\) (Student’s t test). C, wound-healing assay was done with the same cells. Scratches were done on highly confluent cells and closure was monitored by taking pictures at different time points. This assay was repeated three times to confirm results. D, graphs and H&E stainings representing the average number of lung metastases \((P = 0.0239)\) or the total area covered by lung metastases \((P = 0.0416)\) per nude mouse injected with 1 million NMuMG\(^{\text{vprf}}\) vector or p75 CUX1 cells. Statistical significance was determined by the Student’s t test.
Figure 4. Expression and DNA-binding activity of CUX1 transgenes in mammary tumors. A, expression of p75 and p110 CUX1 transgenes in mammary tumors and adjacent normal mammary glands was analyzed by reverse transcription-PCR. GAPDH and cytokeratin 18 are shown as controls. N, adjacent mammary gland; T, mammary gland tumor. B, Immunohistochemical (IHC) staining of mammary tumors from p75 and p110 CUX1 mice using 1300 CUX1 antibodies (epitope map in Fig. 1A). CUX1 protein expression was analyzed by Western blotting using CUX1 1300 and 1062 antibodies (epitope map in Fig. 1A), and DNA binding was assessed in electrophoretic mobility shift assays (EMSA) with double-stranded oligonucleotides containing a consensus binding site for CUX1. D, CUX1 protein expression was analyzed by Western blotting using CUX1 1300 and 1062 antibodies, and DNA binding was assessed by South-Western blotting using double-stranded oligonucleotides containing a consensus binding site for CUX1. Note that a smaller amount of proteins was used from tumor samples to illustrate the increased DNA binding efficiency of CUX1 proteins.
(Fig. 2A; Supplementary Table S1). Mammary tumors developed with an average latency of 20.5 months in 20% and 12% of p75 and p110 CUX1 transgenic lines, respectively, compared with 3% of wild-type FVB/N mice (Fig. 2A; Table 1). In summary, higher CUX1 expression was associated with increased incidence of mammary tumors.

**Mammary tumors are of diverse histologic types.** Histopathologic analysis revealed that mammary tumors were of diverse histopathologic types (Fig. 2B). Some mammary tumors were classified as solid carcinomas with or without papillary differentiation (Fig. 2B, 1 and 2), adenosquamous carcinomas (Fig. 2B, 3 and 4), adenomyoepithelioma (Fig. 2B, 5), or tubular/acinar adenoma (Fig. 2B, 6). Interestingly, adenosquamous carcinomas were most common in the p75 CUX1 line (71% of all breast tumors; Table 1). Two p110 solid carcinomas displayed papillary differentiation; interestingly, one of the two also contained areas of adenosquamous differentiation. One p75 solid carcinoma was described as being cribiform, whereas another one was undifferentiated; in the same mouse, an adjacent mammary gland also contained multifocal squamous nodules. One p75 mouse developed mammary intraepithelial neoplasia, which was not observed in any control mouse (data not shown).

Immunohistochemical staining for cytokeratin 6, 14, and 8/18 confirmed the heterogeneity in histologic phenotypes (Fig. 2C). All tumors included a population of cells staining immunopositive for cytokeratin 8/18, a marker of luminal epithelial cells (Fig. 2C). In addition, in most tumors, we observed populations of cells immunopositive for cytokeratin 14, a marker of myoepithelial cells (Fig. 2C). Moreover, the presence of cytokeratin 6–immunopositive cells in many tumors, particularly in adenosquamous carcinomas and in the adenomyoepithelioma, suggested that these tumors also contained a proportion of progenitor cells (Fig. 2C).

**p75 CUX1 isoform stimulates cell migration and metastasis to the lung.** Metastasis to the lung was observed in three p75 CUX1 transgenic mice with a primary solid carcinoma (Fig. 3A). We detected β-casein mRNA expression in the lung sections from these three mice but not in the lung sections from one mouse that carried a primary lung tumor without a tumor in a mammary gland (Fig. 3A, compare lanes 2-4 with lane 5). One primary carcinoma was undifferentiated (Fig. 3A, p75-452), whereas the second one was cribiform (Fig. 3A, p75-534). The third mouse had two mammary gland tumors in distinct glands: an adenosquamous carcinoma (data not shown) and a papillary mammary gland carcinoma (Fig. 3A, p75-290). It should be noted that the sample size was too small to determine whether the metastasis rate is different between the two lines of transgenic mice. Nevertheless, infection of mammary epithelial NMuMG<sup>TOP2</sup> cells with a retrovirus expressing p75 CUX1 stimulated cell migration in both a two-chamber migration assay and a wound-healing assay (Fig. 3B and C). Importantly, in each case, cells were plated in adjacent wells and counted at the end of the experiments to rule out a proliferation difference (data not shown). Following tail vein injection in nude mice, approximately twice as many metastases were observed in the lungs (P = 0.0239) and the total surface area covered by metastases was four times as large (P = 0.0416) in mice injected with cells expressing p75 CUX1 (Fig. 3D). Together, these results show that p75 CUX1 can stimulate cell migration in tissue culture assays and increases the ability of cells to invade the lung tissue.

**Mammary tumors express an active CUX1 transgene.** The CUX1 transgene mRNA was detected in all mammary tumors and in most of the adjacent normal mammary glands (Fig. 4A). In many cases, transgene expression was higher in the tumor than in the adjacent normal mammary gland. CUX1 proteins were present in the nucleus of tumor cells (Fig. 4B). In some tumors, notably in adenosquamous carcinomas, a clear signal was observed in only a small fraction of tumor cells (Fig. 4B, rightmost). Random chromosome X inactivation would account for the lack of transgene expression in ~50% of the cells. Other reasons, therefore, must account for the detection of transgene in <50% of cells. Because of the low sensitivity of this assay, we cannot exclude that the transgene was expressed below detection level in many cells (Fig. 1C). Another possibility is that the MMTV regulatory sequences were silenced as a result of metaplasia as documented previously (21, 22).

Immunoblotting analysis showed that CUX1 protein expression was higher in the tumor than in the adjacent normal mammary gland (Fig. 4C). In agreement with this, CUX1 DNA-binding activity was higher in tumors than in normal adjacent tissues (Fig. 4C). In some cases, we noted that CUX1 DNA-binding activity appeared relatively higher than what was predicted from the level of protein expression. This was confirmed by performing in parallel immunoblotting and South-Western analyses (Fig. 4D). Importantly, as the South-Western assay involves the separation of proteins in the presence of SDS and their subsequent renaturation, the DNA-binding activity reflects the intrinsic activity of the protein independently of its interactions with various partners in cells. These results suggest that changes in post-translational modifications can contribute to increase the activity of CUX1 transgenes in tumor cells.

**ErbB2 overexpression cooperates with CUX1 in the formation of mammary tumors.** As particular oncogenes tend to cause mammary tumors of definite histologic types, we considered the possibility that different oncogenic pathways cooperated with CUX1 in the development of specific types of mammary tumors (23). To begin to assess this possibility, we analyzed the expression of erbB2 and Wnt genes. Remarkably, erbB2 mRNA and/or protein expression was elevated in many tumors not only in solid carcinomas but also in adenosquamous carcinomas (Fig. 5A). These results suggest that erbB2 overexpression is not obligatorily associated with the development of solid carcinomas and that the pattern of Wnt gene

**Figure 5.** Overexpression of erbB2 in most mammary tumors and activation of the Wnt/β-catenin pathway in adenosquamous carcinomas. A, ErbB2 mRNA and protein expression was measured, respectively, by quantitative real-time PCR (top) and Western blot analysis (bottom) in mammary tumors from p75 and p110 CUX1 transgenic mice. Expression of ErbB2 was calculated as fold difference over adjacent normal mammary gland, adjusted to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels. The proportion of tumors overexpressing ErbB2 is not different between the groups of solid and adenosquamous carcinomas as determined using Fisher’s test (P = 1). B, expression of Wnt genes was measured by quantitative real-time PCR (top) and immunohistochemical staining for β-catenin (bottom) was done in adenosquamous mammary tumors from p75 and p110 CUX1 transgenic mice. Expression of Wnt genes was calculated as fold difference over normal mammary gland obtained from wild-type mice (n = 4), adjusted to GAPDH levels. The same trend was obtained when the fold difference was calculated by comparing tumors with adjacent normal mammary glands from the same mouse (data not shown). C, expression of Wnt genes was measured by quantitative real-time PCR in Hs578T human breast tumor cells. Top, cells carried a lentiviral vector expressing CUX1 specific short hairpin RNA (shRNA) under the control of a doxycycline (Dox)–responsive element. C, no doxycycline; +, treated with doxycycline for 5 d; −, treated with doxycycline for 5 d and then without doxycycline for 5 d. Bottom, cells were transfected with CUX1-specific or scrambled small interfering RNA (siRNA). Expression was measured 3 d after transfection. Bottom right, recruitment of CUX1 to Wnt genes was measured by chromatin immunoprecipitation (ChIP) in Hs578T. D, promoters of the indicated Wnt genes were cloned into a luciferase reporter plasmid. Hs578T cells were transfected with each reporter plasmid together with a control vector or a vector expressing p75 or p110 CUX1. Experiments were done in triplicate and done independently at least three times.
expression may be dominant in specifying mammary tumor types as was reported in bitransgenic MMTV-Wnt1/MMTV-ErbB2 mice (24, 25). As erbB2 overexpression was not observed in all tumors and because short hairpin RNA–mediated knockdown of CUX1 had no effect on erbB2 expression (data not shown), we consider it likely that the increase in erbB2 expression was not induced by CUX1 but rather was caused by molecular events that are independent of CUX1. Interestingly, cytokeratin 18 expression was reduced in many solid carcinomas as reported previously (26, 27).

CUX1 contributes to activate the Wnt/β-catenin pathway in adenosquamous carcinomas. In contrast to the situation in solid carcinomas, in most adenosquamous carcinomas, we observed increased expression of one or more Wnt genes: Wnt6 (in 6 of 6 tumors), Wnt10A (in 5 of 6 tumors), Wnt8B (in 3 of 6 tumors), and Wnt1 (in 2 of 6 tumors; Fig. 5B, top). In agreement with these results, 3 of 3 adenosquamous carcinomas scored immunopositive for β-catenin in the nucleus, whereas no signal was detected in the solid carcinomas tested (Fig. 5B, bottom). The role of CUX1 in the regulation of Wnt gene expression was first suggested from the decrease in Wnt mRNA levels following the knockdown of CUX1 in breast tumor cells using either short interfering RNA or inducible short hairpin RNA expression (Fig. 5C). Chromatin immunoprecipitation showed that CUX1 can bind to the gene promoters of Wnt1, Wnt6, Wnt8b, and Wnt10A (Fig. 5C, bottom). In reporter assays, both p75 and p110 were able to activate expression from the Wnt1, Wnt6, Wnt8b, and Wnt10A gene promoters (Fig. 5D). Altogether, these results indicate that CUX1 proteins bind to the promoters of several Wnt genes and contribute to their up-regulation leading to the activation of the Wnt pathway, which culminates in the presence of β-catenin in the nucleus of mammary epithelial cells.

Discussion

A role for CUX1 in cancer promotion has been suggested from its elevated expression in tumors and cancer cells as well as from its documented effects on cellular processes such as cell cycle progression and cell motility (reviewed in Introduction). Using transgenic mouse models, we provided evidence for the first time that higher expression of short CUX1 isoforms, as observed in breast cancers, contributes to mammary tumorigenesis. Mammary tumors in CUX1 transgenic mice included a wide spectrum of histopathologic types including adenocarcinomas, solid carcinomas with or without papillary differentiation, adenomyoepitheliomas, and tubular or acinar adenomas. Similar ranges of breast tumor subtypes have been observed in transgenic mice expressing other oncogenes such as PY-B, cyclins D1 and D3, prolactin, Wnt1, stabilized β-catenin, LMO4, IRS-1 and IRS-2, insulin-like growth factor-1 receptor, and c-rel (28–39). The presence of many morphologic features of differentiation in tumors from CUX1 transgenic mice suggests that CUX1 can drive tumor development in progenitor cells in a manner that does not preclude their ability to differentiate into committed cells or alternatively that ectopic expression of CUX1 in differentiated mammary gland cells induces them to de-differentiate.

Using site-specific transgenesis, we have compared the ability of p75 and p110 CUX1 to induce tumors in the mammary gland and we noted two important differences (16). First, pulmonary metastasis thus far has been observed in three cases only, all from p75 CUX1 transgenic mice with a primary solid carcinoma in the mammary gland. Although the sample size is still too small to derive any firm conclusion about the metastatic potential of one CUX1 isoform or the other, this finding suggests that it would be worthwhile to carry retrospective studies in human breast cancer cohorts to determine whether the detection of p75 CUX1 protein and/or mRNA in primary tumor specimens may have prognostic value.

Secondly, distinct spectra of mammary tumor subtypes were observed in the two transgenic lines and adenosquamous carcinomas were most common in the p75 CUX1 line (71% of all breast tumors). Interestingly, the accelerated ductal elongation observed in p75 CUX1 mice resembles what was reported in the Wnt1 transgenic mouse model, and the range and types of mammary tumors observed in the p75 CUX1 mice resemble those induced following activation of the Wnt/β-catenin pathway (32, 39–41). In Drosophila, several genetic interactions have been documented between Notch, Wingless, and Cut (reviewed in ref. 41). Mutations that affect the expression or function of Cut, Wingless, or Notch during wing development were found to produce closely related phenotypes (42). Subsequent studies suggested a regulatory cascade whereby activation of Notch in cells of the dorsal/ventral boundary results in the induction of Cut, which in turn functions to maintain Wingless expression (43–45). In the present study, we provided biochemical evidence to implicate mammalian homologues of Cut in the regulation of several Wnt genes (Fig. 5B–D). CUX1 was shown to bind to the 5′-flanking sequences of several Wnt genes (Fig. 5C); CUX1 mRNA knockdown caused a decrease in Wnt gene expression (Fig. 5C); in reporter assays, both p75 and p110 CUX1 were able to activate Wnt gene promoters (Fig. 5D). We speculate that the differences in tumor subtypes reflect the relative capability of p110 and p75 CUX1 to regulate Wnt genes. This may be due to the fact that the p75 isoform binds more stably to DNA (6) or that it is better suited to form a complex with other factors that participate in the regulation of Wnt genes.

It is important to stress, however, that although CUX1 is required for Wnt gene expression, overexpression of CUX1 does not appear to be sufficient alone to activate these genes. Indeed, Wnt gene expression in different stages of mammary cycle development did not correlate well with that of the CUX1 transgene (Fig. 1B and C; data not shown). Moreover, the latency of tumor development in CUX1 transgenic mice was longer, and the penetrance was lower, than in Wnt transgenic mouse models (reviewed in ref. 46). Clearly, the long latency period and incomplete penetrance in CUX1 transgenic mice indicates that other oncogenic events must take place to allow for tumor development. The overexpression of several Wnt genes in the mammary tumors from CUX1 transgenic mice suggests that at least some of these transforming events must cooperate with CUX1 in the transcriptional activation of Wnt genes. Future studies shall aim to identify the other players that participate with CUX1 in the regulation of Wnt genes.

Note Added in Proof

Since submission of this article, five more mammary tumors were observed in p110 CUX1 transgenic mice (23 and 24 month old), one in p75 CUX1 mice (22 month old), and none in control littermates.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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