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Compartment Switching of *WNT-2* Expression in Human Breast Tumors

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Abstract

WNT-2 is a secreted polypeptide with mitogenic effects in murine mammary epithelial cells, but its role in human cancer is unknown. Using RNase protection analysis of primary cell preparations and *in situ* hybridization analysis, we report that *WNT-2* is expressed at low levels in normal human breast fibroblasts but not in epithelial cells. *WNT-2* was found to be expressed at high levels in both the epithelium and stroma of 5 of 11 infiltrating carcinomas and 2 of 6 fibroadenomas. The high level of *WNT-2* expression in tumor epithelium suggests that tumorigenesis may involve the ectopic expression of WNT-2 and the creation of an autocrine Wnt signaling loop.

Introduction

The prototype member of the *Wnt* gene family, *Wnt-1,* was first isolated as a common site of integration by the mouse mammary tumor virus and was later shown to be homologous to the *Drosophila* segment polarity gene *wingless* (1). Members of the *Wnt* gene family are highly conserved through evolution (reviewed in Ref. 2) and have been implicated in the control of numerous developmental processes such as *Xenopus* axis formation (3) and murine hindbrain and kidney development (4–6) Biochemical studies of Wnt proteins in mammalian cell culture and in *Drosophila* have shown that Wnt polypeptides are glycosylated, secreted, and associate with the cell surface/extracellular matrix (7–9). Several members of the *Wnt* gene family, including *Wnt-1* and *Wnt-2,* share the ability to induce cell proliferation in mammary epithelial cells by an autocrine or paracrine route (10, 11). This effect may be mediated by the ability of Wnt-1 to regulate free pools of intracellular catenin proteins, which then form stable complexes with E-cadherin or the tumor suppressor gene product adenomatous polyposis coli (12, 13).

In mouse mammary epithelial cells *in vivo*, mouse mammary tumor virus integration activates *Wnt-1* by increasing transcription of *Wnt-1* mRNA, leading to increased (tumorigenic) levels of the normal Wnt-1 protein. Ectopic expression of Wnt-1 *in vivo* has been achieved using transgenic mice and recombinant retroviruses. In both systems, Wnt-1 induced the formation of a hormone-independent hyperplasia with a feathery morphology (14, 15). Although Wnt-1 was not expressed in the normal gland, several other members of the *Wnt* gene family are expressed during mammary development (16, 17). *Wnt-2* expression was detected in glands from virgin mice in four of five strains of mice examined and was found to be down-regulated during pregnancy (16, 18).

Several members of the *Wnt* gene family have been found to be expressed in human breast cell lines and in normal and diseased states of human breast tissue. Preliminary results from surveys of *Wnt* expression have suggested that *WNT-2*, *WNT-4*, *WNT-5A*, and *WNT-7B* may be associated with abnormal proliferation in human breast tissue (19). More detailed studies have confirmed an association of *WNT-5A* expression with benign and invasive cancer (20–22). In this study, we found overexpression of *WNT-2* in 5 of 11 infiltrating carcinomas, and in each case, we detected expression in the epithelium. The initiation of *WNT-2* expression in the epithelium of some carcinomas and two fibroadenomas with epithelial hyperplasia suggests that this change may be a component of breast epithelial hyperplasia.

Materials and Methods

Cell Separations. Primary human breast epithelial cell cultures were prepared as described by Clarke *et al.* (23). Briefly, human breast tissue obtained from reduction mammoplasties was cut into small pieces, digested with collagenase, and passed through graded filters to collect the fibroblasts as single cells and the ducts and lobules (organoids), which are washed from the filters. The fibroblasts are collected from the filtrate (through a 53 mM sterile nylon filter) and grown in DMEM with 10% FCS (24). The epithelial cells are prepared by plating out the organoids onto plastic for 7–10 days, when the myoepithelial cells mobilize and spread out on the plastic with luminal cells on top. At the end of this period, the luminal and myoepithelial cells can be separated into individual populations using an immunomagnetic separation technique (23). This method is dependent upon the exclusive expression of the common acute lymphoblastic leukaemia antigen (CALLA/CD10) on myoepithelial cells and the presence of the epithelial membrane antigen on luminal cells. The purity of the populations is determined by immunocytochemical analyses of plated cells with monospecific cytokeratin antibodies (cytokeratins 18 and 19 as markers of luminal cells and cytokeratin 14 for myoepithelial cells). Using these procedures, we were able to prepare luminal and myoepithelial cell cultures of 95–99% purity. Separated cells were grown as described previously (25); after 7 days, the cells were harvested and immediately placed in guanidinium thiocyanate-phenol-chloroform extraction buffer.

Tumors and Tissues. The tissue used for the *in situ* hybridization studies was taken from the routine archival pathology material at the Royal Marsden Hospital. This tissue is placed immediately in buffered formal saline at the time of surgery. We selected tumor blocks that had both infiltrating carcinomas and adjacent normal tissue on the same section. Six fibroadenomas, six cases of benign epithelial hyperplasia (including a ductal adenoma, duct ectasia, and apocrine cysts), and six normal breast samples from reduction mammoplasties were also studied. Tumors were graded according to the United Kingdom national breast screening guidelines (26).

***In Situ* Hybridization.** Prior to analysis for ***WNT-2* expression, sections from each sample were assessed for the presence of mRNA by hybridization to** ^{33}P-end labeled oligo(dT(30)). Subsequent experiments were then performed only with samples that contained significant levels of mRNA to eliminate false-negative results. *In situ* hybridization with ^{33}P-labeled oligonucleotides was performed as described previously (16). Ten 30-mer antisense oligonucleotides were designed against the human WNT-2 sequence: ccagagccagattccaccgagaggggcgtt; gggggtgaagccaggtcaagagcagagggag; tctcatgtaaccaccatgaagagttgacctc; gcacatcaccctggaggagccacctgtagc; tgctgtccactcggccacgccctggctaat; aaggctgtgatccctgtccagggtgttgca; aaaaatgcctttgctgctgtccttggcgcttcc; aaaaatgcctttgctgctgtccttggcgcttcc; cacagtgaaacctgtgccatcctggttcat; and tgtagcggttgtccagtcagcgttcttggg. Control hybridizations were performed with a mixture

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of 30-mer oligonucleotides that contained all four bases at each position p(dN(30)). Exposure times were for 8 weeks.

RNase Protection. RNA was prepared from tissues by acid guanidium thiocyanate phenol chloroform extraction, as described by Chomczinski and Sacchi (27). RNase protection was performed by standard protocols using constructs described previously (19). The full-length WNT-2 probe was 487 base pairs and can be distinguished from its protected fragment of 389 base pairs. In all assays, 10 mg of RNA were hybridized to a cocktail containing 10^5 cpm of each of the WNT-2 and control glyceraldehyde-3-phosphate dehydrogenase probes.

Results

***WNT-2* Is Normally Expressed in Breast Fibroblasts.** The *in situ* hybridization studies showed a positive but weak signal at the epithelial/stromal junction in 1 case of 10 normal breast samples (Table 1; Fig. 1). Because of the resolution achievable with the ^{33}P-labeled probes, it was not possible to define if the signal was coming from the fibroblasts in the intralobular stroma or the epithelium. However, sensitive RNase protection studies indicated that *WNT-2* expression could be detected in the majority of normal breast samples (Fig. 2, *Lanes N*; Ref. 19). To localize *WNT-2* expression in the normal breast, primary epithelial and fibroblast cell populations were first separated and then analyzed by RNase protection. The primary cells were prepared from reduction mammoplasties using a technique that routinely generates >90% pure cell populations as judged by immunohistochemistry for cell type-specific markers (23). Four of four breast cell preparations showed expression of *WNT-2* in the mammary fibroblasts but not in the mammary epithelial cells. *WNT-2* mRNA levels in the purified fibroblasts were comparable to those in the total breast material from which the cells were derived. An analysis of purified primary luminal epithelial cells (myoepithelial cells and fibroblasts were not present) also failed to detect *WNT-2* expression (data not shown). We conclude that *WNT-2* is expressed at low levels in mammary fibroblasts.

***WNT-2* Expression in Benign and Malignant Breast Biopsies.** *WNT*-2 expression in a panel of formalin-fixed, paraffin-embedded breast tissues was analyzed by *in situ* hybridization. Two of 11 infiltrating carcinomas had very high levels of *WNT-2* expression in the tumor epithelium and stroma (Fig. 1; Table 1, +++; ductal carcinomas), whereas another 3 tumors had levels of *WNT-2* that were significantly above background (Fig. 1; Table 1, +; ductal carcinomas). Very weak and nonexpressing tumors included four lobular carcinomas and two ductal carcinomas. Two of six fibroadenomas had significant levels of *WNT-2* expression in the epithelial and stromal components. These two lesions were characterized by their hyperplasia of the epithelial component in addition to the more normal stromal component. None of the other benign or normal tissues showed strong *WNT-2* expression (Table 1). A direct comparison of *WNT-2* expression in tumor and normal tissue showed high levels of *WNT-2* in the tumor, whereas levels in the adjacent normal tissue were undetectable (Fig. 1*B*, normal tissue shown by *arrow*). Levels of *WNT-2* expression (Fig. 1*A* and Fig. 2, *Tumor *ID*) were detected by both RNase protection and *in situ* hybridization.

Because amplification of the murine *Wnt-2* gene has been observed in two murine tumors derived from the GR strain of mice (28), we tested for *WNT-2* amplification in a range of breast cancers including three samples whose *WNT-2* expression was equivalent to the tumor ID[2] (Fig. 2). Southern analysis showed that 15 of 15 tumors had a normal copy number of *WNT-2* and showed no evidence of *WNT-2* rearrangement (data not shown). Slot blot analysis of DNA from tumor *ID also revealed a normal DNA copy number. This suggests that the increase in *WNT-2* expression does not involve gene amplification.

Table 1 *Wnt-2 expression*

Sample type	Sample	Stroma[a]	Epithelium[a]
Infiltrating carcinoma	A	+	+
n = 11	B	±	±
	C	+	+
	D	−	−
	E	−	−
	G	−	−
	I	+++	+++[b]
	J	+	+
	K	±	±
	L	+++	+++[b]
	M	−	−
Fibroadenoma n = 6	A	−	−
	B	±	±
	C	±	±
	D	+	+[c]
	E	++	++[c]
	F	−	−
Benign (n = 6)		−	−
Normal gland[d] n = 10	A	−	−
	B	−	−
	C	−	−
	D	+[e]	+[e]
	E	−	−
	F	−	−
	G	−	−
	H	−	−
	I	−	−
	J	−	−

[a] ±, very weak; +, low; ++, medium; +++, very high.
[b] See Fig. 2.
[c] Patches of positive cells throughout section.
[d] Samples G–J were measured on blocks of material that also contained infiltrating carcinomas.
[e] Differentiation between epithelium and stroma difficult due to low level expression and silver grains over closely apposed epithelium and stroma.

Discussion

Breast Tumors Express *WNT-2*. In support of previous studies, we found expression of *WNT-2* in normal, benign, and malignant breast tissues (19). To understand the functional significance of these observations, we investigated the location of *WNT-2* message. We expected to find a correlation in *WNT-2* mRNA levels with the proportion of cells that expressed *WNT-2* in the normal breast. Unexpectedly, we found that *WNT-2* was expressed in the epithelium of 5 of 11 infiltrating ductal tumors but was present exclusively within the fibroblasts of the normal breast. *WNT-2* expression in the primary fibroblasts probably reflects the site of *WNT-2* expression *in vivo* as human mammary epithelial and fibroblast cells retain many of their differentiated markers in culture (23, 24). In addition, murine *Wnt-2* expression was detected in the stromal fibroblasts but not in the epithelium of 6-week-old virgin animals by *in situ* hybridization (18). In a separate study, mRNA from epithelial-free mouse mammary fat pads was shown to contain high levels of *Wnt-2* message (16). In combination with the results described here, these data strongly argue that the stroma is the normal site of *Wnt-2* expression in the resting mammary gland. Similar studies of *Wnt-2* expression in the lung showed expression exclusively in the stroma (29), suggesting that Wnt-2 may have a conserved role within the stroma of ductal/alveolar structures.

Because WNT-2 has a mitogenic effect on mouse mammary epithelial cells (10), it is reasonable to propose that stromal expression of the WNT-2 ligand may control epithelial cell proliferation. Under these circumstances, the finding that *WNT-2* is expressed in the epithelium of a proportion of breast tumors and fibroadenomas raises

[2] The abbreviation used is: ID, infiltrating ductal carcinoma.

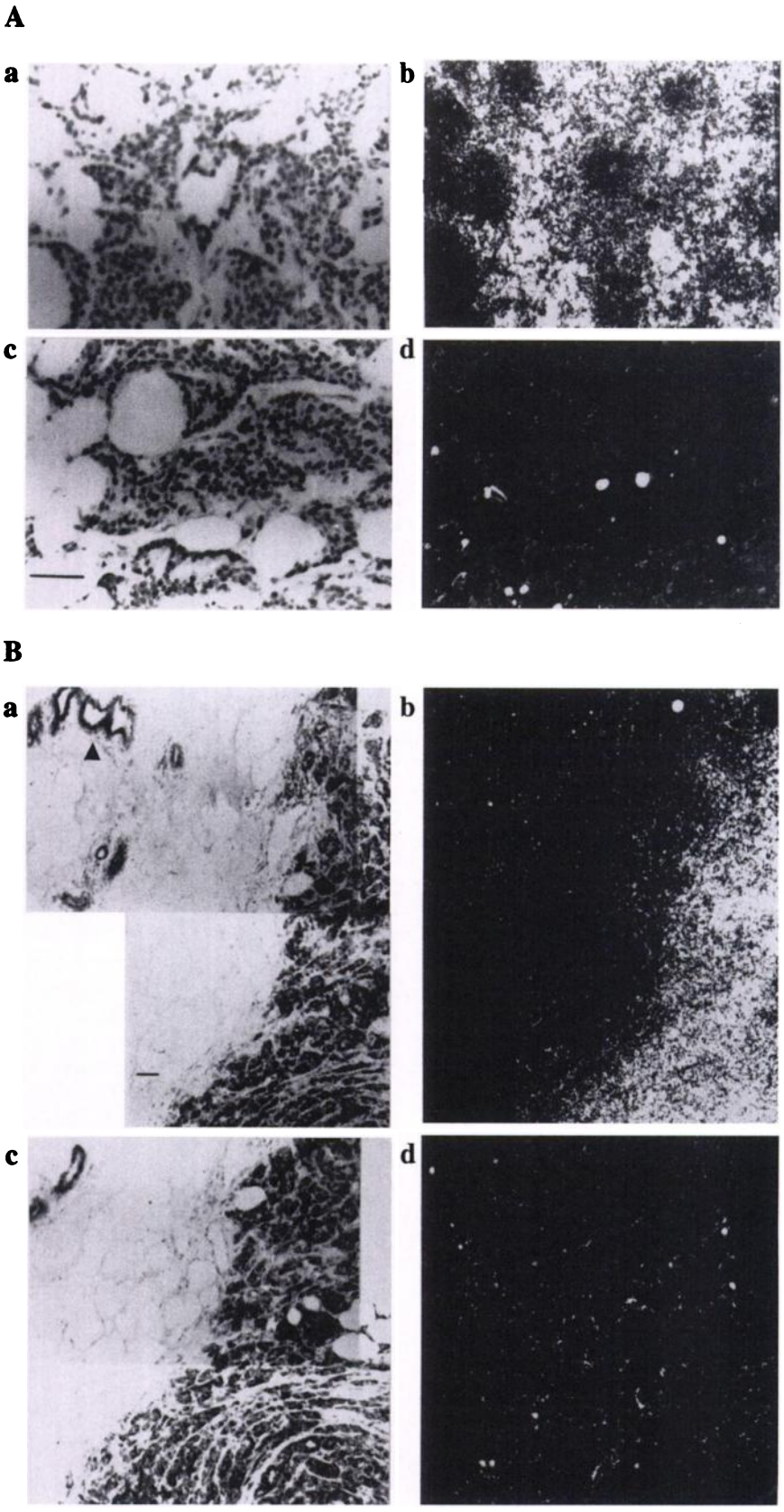

Fig. 1. *In situ* hybridization for *WNT-2* expression in breast carcinomas. *A* and *B*, infiltrating ductal carcinomas samples I and L, respectively (see Table 1). *a* and *c*, light field micrographs; *b* and *d*, corresponding dark field micrographs. *a* and *b*, hybridization with WNT-2 probe; *c* and *d*, hybridization with control probe to the next serial section. *Arrow head*, normal duct. *Bar*, 100 μM.

the possibility that WNT-2 expression and reception within the epithelial cells creates an autocrine signaling loop that may deregulate normal epithelial proliferation in some fibroadenomas and ductal carcinomas. Although the high levels of *WNT-2* within the tumor stroma may relate to the pattern of *WNT-2* expression in the normal tissue, the higher levels of expression within the epithelium could be the result of the aberrant initiation of expression within epithelial cells or may be due to the clonal expansion of a subpopulation of cells that

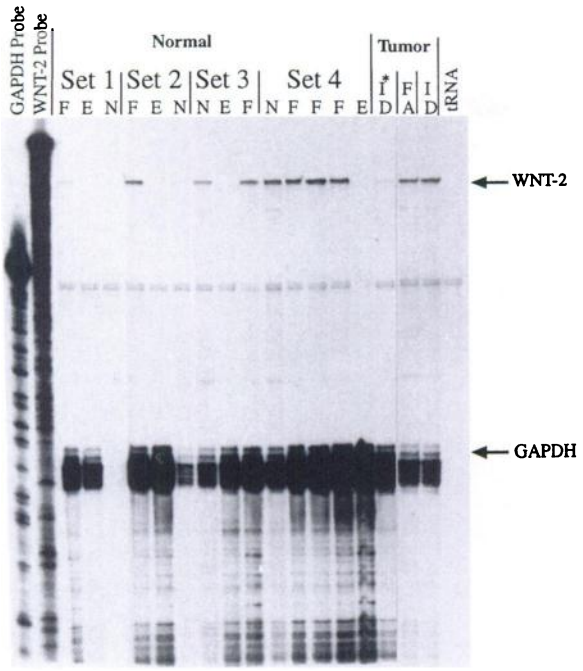

Fig. 2. Analysis of WNT-2 expression in normal breast tissue and matched primary cells. Expression of WNT-2 in four matched sets of tissues and corresponding separated cells. *F*, fibroblast cells; *E*, epithelial cells; *N*, normal breast from reduction mammoplasty prior to cell preparation. *Tumors: ID*, infiltrating ductal carcinoma; **ID*, sample corresponding to *in situ* hybridization shown in Fig. 1*A* (*a*). *FA*, fibroadenoma.

normally express *WNT-2*. In this context, the observation that some epithelial cells within murine ductal end buds express *Wnt-2* suggests that *Wnt-2* expression could be a characteristic of this developmentally distinct, highly proliferative cellular population (18). An alternative or possibly additional mechanism to account for the high level of *WNT-2* expression in the breast tumors is suggested by the observation that *Wnt-2* was amplified in murine tumors derived from the GR strain of mice (28). However, Southern analysis of a panel of breast tumors suggested that this is unlikely to be a standard mechanism of *WNT-2* expression in human tumors. Overall, these data suggest that the WNT-2 protein is operational in the stroma of the normal gland and when expressed in tumor epithelium may contribute to the maintenance and possibly the generation of the transformed phenotype.

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