**Wnt5a Cloning, Expression, and Up-Regulation in Human Primary Breast Cancers**

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**ABSTRACT**

Wnt genes are involved in mouse mammary cancer, but their role in human cancer is unknown. Human Wnt5a was cloned from a placental cDNA library and used to assess expression by ribonuclease protection and *in situ* hybridization in human breast cell lines and normal, benign, and malignant breast tissues. Human Wnt5a shows over 99% homology at amino acid level with mouse Wnt5a, and 90% with *Xenopus* Wnt5a. It was expressed only at low levels in breast cell lines and normal breast tissue. Benign proliferations and invasive cancer respectively showed 10-fold and 4-fold higher Wnt5a than normal breast tissues. The greater up-regulation in benign conditions suggests a role in aberrant differentiation. *In situ* hybridization localized the signal to the epithelial component. Wnt5a is the first member of the Wnt family to demonstrate overexpression in human breast cancer. It was not associated with factors known to affect breast cancer prognosis such as lymph node status or epidermal growth factor receptor status.

**INTRODUCTION**

In mouse mammary tumor virus-induced breast cancer, analysis of insertion sites has shown activation of endogenous genes, *int* genes (1). *Int-1* (now *Wnt1*) was the first gene isolated and shows strong homology to a *Drosophila* developmental gene, wingless (2), involved in pattern development. Two other members of this family, Wnt3 and Wnt2 (also called int-related protein, irp) are also involved in mouse mammary cancer (3, 4).

Fifteen Wnt genes have been isolated in vertebrates. They are expressed in many adult and embryonic tissues (5) and are involved in morphological development. The importance of their role is reflected by the severity of the phenotypic abnormalities that result from aberrant Wnt expression. Thus, Wnt genes have important roles in development and in cancer.

The role of Wnts in mouse mammary carcinogenesis has been extensively studied and there is evidence that they are secreted proteins, processed via the Golgi apparatus (6), which remain tightly associated with the extracellular plasma membrane or matrix (7). Wnts produce morphological effects on some mouse mammary cancer cell lines by transfection in autocrine (8) and paracrine mechanisms (9).

A survey of expression in normal mouse mammary gland development showed that some Wnts are expressed in virginial breast, some in pregnancy, and others in lactation (10). However, *Wnt1*, which is involved in carcinogenesis is not expressed in normal mouse mammary tissue. This implicates Wnt gene family members in normal breast development and suggests aberrant expression of other members can contribute to malignancies (11).

Evaluation of the normal expression of Wnt genes in human breast epithelium and cancer would contribute to understanding the role of Wnt genes in human cancer. It has been shown that some of the Wnt genes are expressed in human breast tissue, and that quantitative differences exist in the Wnt expression profile of normal and proliferative lesions (12).

We chose Wnt5a as a candidate human gene to clone and evaluate because it is expressed in normal mouse breast epithelium to a low extent, and also in mouse breast cancer cell lines (10). Furthermore it has some different properties from the human Wnt genes previously cloned in its effects on cell gap junctions (13) and *Xenopus* development (14). Interactions between Wnt genes with different normal functions may contribute to malignant transformation (11).

**MATERIALS AND METHODS**

**Isolation of 384-Base Pairs Fragment of Human Wnt5a from Fetal Brain cDNA Library.** Two hundred ng of a human fetal brain library in plasmid pCDM8 (obtained from Dr. D. Simmons and Dr. J. Fawcett, Institute of Molecular Medicine, Oxford, United Kingdom) was used as a template for PCR. Amplification of cDNA was carried out using 500 ng of each of the degenerate forward (5'-GGGGAATTCACGAGA/GCTGCGTG/) cAA/GTG/CCAT-3') and reverse (5'-AAATCTAGAGA/
GCAGCATCACTG/TG/GG-3') oligonucleotide primers previously described by Gavin et al. (5). PCR products were separated on a 2% agarose gel. Products of the correct size (predicted from known Wnt sequences) were recovered, then further amplified using the above primers and ligated into the plasmid pBluescript KS+ (Stratagene). JM109 cells were transformed with the reaction products, and the nucleotide sequences of clones containing inserts of correct size were determined by dideoxy chain termination sequencing. Clones with significant homology to known *Wnts* were identified using the FASTA program (CGC) and included one clone containing a 384-base pair fragment of human Wnt5a.

**Isolation of Human Wnt5a from Placental cDNA Library.** Replica colony lifts of approximately 10^7 recombinant clones of a human placental library in the plasmid pCDM8 were prepared using Hybond-N membranes (Amersham). The membranes were hybridized to a Wnt5a probe synthesized using the

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1 This work was funded by the Imperial Cancer Research Fund and Oxfordshire Health Authority.

2 To whom requests for reprints should be addressed.
Wnt5a Up-Regulation in Human Breast Cancer

384-base pair fragment isolated as described above. The probe was generated by incorporating [α-32P]dCTP (Amersham) in a PCR amplification of the 384-base pair fragment using the above degenerate primers. The resultant species were end filled and separated from unincorporated nucleotides by passage through a Sephadex G-50 spin column (Boehringer Mannheim). After hybridization of the probe to the membranes, positive clones were identified by autoradiography, and subjected to further rounds of screening. After four rounds of screening, positive clones were selected and sequenced. Clones with significant homology to known Wnts were identified and included one containing 798 base pairs of the 3' end of the human Wnt5a cDNA as well as some 3' untranslated sequences.

Further sequence 5' to the 798-base pair 3' terminus was obtained using a nested PCR strategy. A primary PCR reaction was carried out using 200 ng of a placental cDNA library in pCDM8 as template. The primers used were a mouse Wnt5a-specific forward primer FP1 (5'-ATGAAGAAGCCCCATTGG-GAATA-3') corresponding to the 21 extreme 5' nucleotides of mouse Wnt5a, and a human Wnt5a-specific reverse primer RP1 (5'-GAACGCCGCCGCTATGGGTT-3') corresponding to a known sequence in the 798-base pair partial clone. Fragments of correct size (approximately 462 base pairs) were recovered and used as template for nested PCR. In the nested PCR reaction the forward and reverse primers (FP2 and RP2) were 3' and 5' of the primers FP1 and RP1, respectively. Primer FP2 (5'-AATCTNGGTGTNCNGTG-3') was a fully degenerate primer, and primer RP2 (5'-GTGTTATCCACAGTGCT-3') was a human Wnt5a-specific primer corresponding to the extreme 5' region of the 798-base pair partial clone. Fragments of correct size (approximately 234 base pairs) were recovered, subcloned into plasmid pBlueScript SK, and sequenced. Clones with significant homology to Wnt5a were identified and included one containing 234 base pairs of human Wnt5a sequence 5' to the 798-base pair partial clone.

A nested PCR strategy was also used in order to clone the extreme 5' region of Wnt5a. In a primary PCR reaction, 200 ng of human placental cDNA library in pCDM8 was used as template. The primers used were a pCDM8-specific primer and the human Wnt5a-specific internal primer RP1. Products of a large enough size to contain the 5' end of Wnt5a were recovered and used as template in the nested PCR. In the nested PCR reaction, primers FP1 and RP2 were used. Fragments of correct size (approximately 366 base pairs) were recovered, cloned into TA cloning vector (Invitrogen), and sequenced. A clone containing the 5' end of Wnt5a was identified. Thus the sequences of the original 798-base pair partial clone and the two nested PCR products combined to give the full-length sequence.

Chromosomal Localization of Human Wnt5a. Twenty-μg DNA samples from a panel of human-hybrid cell lines, and control human, mouse, and hamster DNA (obtained from Dr. N. Spurr, Imperial Cancer Research Fund, London, United Kingdom) were digested with EcoRI, fractionated on a 0.7% agarose gel, and transferred to Hybond-N membranes according to Southern’s protocol (15). The membranes were then hybridized to a random primer generated [α-32P]dCTP-labeled probe, using a 1.4-kilobase XbaI fragment of the original partial human Wnt5a clone as template. Signals were detected by autoradiography.

Ribonuclease Protection Assays. Ribonuclease protection assays were carried as described in Ref. 16. Briefly, a 384-base pair fragment of human Wnt5a was cloned into pBlue Script KS. The EcoRV linearized plasmid was used to generate antisense [α-32P]CTP-labeled probes with T7 RNA polymerase (Gibco). Probes were hybridized to 10-μg samples of total RNA extracted from tissues and cell lines by a single-step extraction method (17). Hybridization was carried out at 45°C for 16 h. Each hybridization also contained an antisense probe for GAPDH3 as a loading control. GAPDH probes were prepared from a 120-base pair fragment of GAPDH cloned into pBlue Script SK (18). Unhybridized probe was digested with RNase A and T1, and protected fragments were electrophoresed on polyacrylamide gels. Dried gels were autoradiographed.

Image Analysis. Autoradiographs of ribonuclease protection assays were scanned using a Bio-image analyser (Milligan Bioneresearch) to determine RNA abundance. Wnt5a values were normalized to GAPDH to allow for loading. MCF10 RNA was included in all assays as a positive control. This was assigned a unit level of expression and all other values were standardized to this.

Cell Lines. The following breast cell lines were obtained from ATCC (Bethesda, MD): T47D (ATCC HTB133), MDA231 (ATCC HTB26), MCF10 (ATCC CRL10317), MDA415 (ATCC HTB128), MDA453 (ATCC HTB31), MDA157 (ATCC HTB24), BT20 (ATCC HTB19), SKBR3 (ATCC HTB30), ZR9811 (ZR-75-1), ZR4, and ZR11 were obtained from Dr. E. Valvenius (Department of Pathology, University Hospital, Uppsala, Sweden). MCF7s were obtained from Dr. B. Durkacz (Cancer Research Unit, University of Newcastle upon Tyne). Adriamycin-resistant MCF7s were obtained from Dr. R. Cowen (National Institutes of Health). Lines 2-5-2a, 3-4-1, 5-3-1, 6-1-1, MTSV1-7, and MTSV4-1 were obtained from Dr. J. Taylor (Imperial Cancer Research Fund, London, United Kingdom).

Cell Culture. T47D, MDA231, MCF10, MDA453, SKBR3, MCF7, Adriamycin-resistant MCF7, ZR9811, ZR4, and ZR11 were maintained in DMEM with 10% FCS. BT20 was maintained in Eagle’s MEM supplemented with 15% FCS and 2 mm glutamine. MDA415 was maintained in DMEM, 15% FCS, 1 μm hydrocortisone, 10 μg/ml insulin, and 10 μg/ml glutathione. MDA157 was maintained in RPMI 1640 and 10% FCS. MTSV1-7, MTSV4-1, 2-5-2a, 3-4-1, 5-3-1, and 6-1-1 were maintained in DMEM: Ham’s F12 (1:1), 10% FCS, 10 μg/ml insulin, and 5 μg/ml hydrocortisone. All cultures were grown on plastic dishes in 5% CO2-95% air in humidified incubators. All cultures were free of Mycoplasma.

Handling of Clinical Samples. Protocols for handling of clinical samples and assays for hormone and growth factor receptors were followed as detailed in LeJeune et al. (19). Briefly, Tumors were considered to be ER positive if they contained at least 10 fmol of specific binding sites per mg of cytosolic protein, and EGFR positive if they contained at least

The abbreviations used are: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ER, estrogen receptor; EGFR, epidermal growth factor receptor; ATCC, American Type Culture Collection.
20 fmol of specific binding sites per mg of membrane protein. Human tissue samples were selected to represent normal breast tissue, benign breast disease, and breast cancer.

In Situ Hybridization. A single-stranded antisense RNA probe to Wnt5a was transcribed from EcoRI-linearized Wnt5a DNA using T3 DNA polymerase (Promega), and 35S-UTP (~800 Ci/mmol; Amersham International) as the sole source of UTP. Histological sections of human tissues that had been fixed in neutral-buffered formalin and embedded in paraffin wax were treated in the manner described by Senior et al. (20) with minor modifications. In summary, 1 x 10^6 cpm of unhydrolyzed probe in 10 ml buffer was hybridized overnight at 55°C to sections permeabilized with proteinase K. Posthybridization steps included several large volume washes in a 50% formamide buffer at 55°C to remove unhybrized probe, RNase A treatment to remove these cleaved fragments were included several large volume washes in a 50% formamide buffer at 55°C to remove unhybrized probe, RNase A treatment to digest single-stranded and imperfectly hybridized domains, and extensive washing to remove these cleaved fragments were performed. The final washes were in 0.5X SSC (1X SSC is 0.15 M NaCl and 0.015 M sodium citrate) at 65°C for 30 min twice. Slides were dehydrated and processed for autoradiography (Ilford K2) at 4°C for 7 to 10 days. Latent images were developed with Kodachrome (a!.

Levels of expression measured by nuclease protection assays were low, requiring 7 days of development. One line and Fig. 3). Levels of expression measured by nuclease protection assays were low, requiring 7 days of development. One line

RESULTS

Isolation of Wnt5a cDNA. A partial length cDNA was initially isolated by using PCR primers to homologous domains in Wnt genes. This was used to isolate a cDNA from a human placental library, which, in combination with nested PCR products from the same library provided the full human Wnt5a cDNA coding sequence shown in Fig. 1 with the corresponding amino acid sequence. The sequence comparison of human Wnt5a with mouse and Xenopus Wnt5a shows extensive conservation with amino acid level homology of 99% and 90%, respectively.

Chromosomal Localization of Wnt5a. The chromosome location of the human Wnt5a was determined by screening hamster-human and mouse-human hybrid cell lines which had known human chromosome karyotypes. Southern blotting showed a Wnt5a signal located on human chromosome 3 (Fig. 1K2). Southern blotting showed a Wnt5a signal located on human chromosome 3 (Fig. 2). Thus, in vitro cell lines rarely expressed Wnt5a.

Expression in Human Breast Tissue. Expression was assessed in human breast tissue, tissue from benign breast diseases, and primary breast cancers. The clinical details of the patients (e.g., age, tumor receptor status) are given in Table 2.

in all but one case (BT20). Thus, in vitro cell lines rarely expressed Wnt5a.

Expression in Human Breast Cell Lines. To assess expression in human breast cancer, a panel of human breast cell lines was initially analyzed, since they represent a pure epithelial population. Cell lines from normal breast duct luminal epithelium, benign epithelial proliferation, and in situ or invasive components of breast epithelium were studied (Table 1 and Fig. 3). Levels of expression measured by nuclease protection assays were low, requiring 7 days of development. One line from luminal epithelium had higher levels than the others.

Cell lines established from malignant pleural effusions and metastasis had lower levels than the luminal cell line MTSV1-7
Wnt5a Up-Regulation in Human Breast Cancer

Southern blot of human-rodent hybrid cell lines with human Wnt5a probe. Lane 1, human genomic DNA; Lane 2, mouse genomic DNA; Lane 3, hamster genomic DNA. Lanes a-r, DNA from hybrid human-rodent cell lines, each bearing human chromosome DNA. Lanes: a, chromosome 1; b, chromosome 2; c, chromosome 3; d, chromosome 5; e, partial chromosome 6; f, chromosome 7; g, chromosome 8; h, chromosome 9; i, chromosome 11; j, chromosome 12, k, chromosome 13; l, chromosome 14; m, chromosome 15; n, chromosome 16; o, chromosome 17; p, chromosome 19; q, chromosome 20 and partial 4; r, chromosome 21. Arrows in Lanes 1 and c, signal for human genomic DNA (Lane 1) corresponds to that in Lane c, i.e., chromosome 3. The absence of Wnt5a signal in a human-mouse hybrid cell line carrying a p terminus deleted chromosome 3 (data not shown) suggests that Wnt5a is on human chromosome 3p.

Table 1
Comparison of Wnt5a expression in breast cell lines derived from ductal epithelium, in situ and invasive components of breast carcinoma, and breast cancer metastases

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Type</th>
<th>Expression standardized to MCF10</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF10</td>
<td>Immortal nonmalignant</td>
<td>1</td>
</tr>
<tr>
<td>ZR9B11</td>
<td>ER+ breast cancer</td>
<td>0</td>
</tr>
<tr>
<td>ZR11</td>
<td>ER+ breast cancer</td>
<td>&lt;1</td>
</tr>
<tr>
<td>ZR4</td>
<td>ER+ breast cancer</td>
<td>0</td>
</tr>
<tr>
<td>T47D</td>
<td>ER+ breast cancer</td>
<td>0</td>
</tr>
<tr>
<td>MCF7</td>
<td>ER+ breast cancer</td>
<td>0</td>
</tr>
<tr>
<td>MDA231</td>
<td>ER- breast cancer</td>
<td>1.8</td>
</tr>
<tr>
<td>MDA415</td>
<td>ER- breast cancer</td>
<td>0</td>
</tr>
<tr>
<td>MDA453</td>
<td>ER- breast cancer</td>
<td>0</td>
</tr>
<tr>
<td>MDA157</td>
<td>ER- breast cancer</td>
<td>0</td>
</tr>
<tr>
<td>BT20</td>
<td>ER- amplified EGFRI</td>
<td>0.0012</td>
</tr>
<tr>
<td>SKBR3</td>
<td>ER- amplified erbB-2</td>
<td>0</td>
</tr>
<tr>
<td>Adriamycin-resistant MCF7</td>
<td>Drug-resistant MCF7</td>
<td>0</td>
</tr>
<tr>
<td>2-5-2A</td>
<td>Derived from benign</td>
<td>0</td>
</tr>
<tr>
<td>component of breast</td>
<td>component of breast</td>
<td></td>
</tr>
<tr>
<td>3-4-1</td>
<td>Derived from in situ</td>
<td>1</td>
</tr>
<tr>
<td>5-3-1</td>
<td>Derived from in situ</td>
<td>1</td>
</tr>
<tr>
<td>6-1-1</td>
<td>Derived from in situ</td>
<td>0.0004</td>
</tr>
<tr>
<td>MTSV1-7</td>
<td>Luminal normal cells, SV40</td>
<td>0.0012</td>
</tr>
<tr>
<td>MTSV4-1</td>
<td>Luminal normal cells, SV40</td>
<td>0.0012</td>
</tr>
</tbody>
</table>

Normal tissues (n = 15) were obtained either from reduction mammoplasties (n = 7), or from normal tissue adjacent to tumors (n = 8). Benign breast disease samples consisted of fibroadenomas (n = 5), fibrocystic disease (n = 3), and benign phyllode tumors (n = 2). Tumors (n = 28) were selected to represent different subgroups according to known prognostic factors (node, ER, and EGFR status). In contrast to the cancer cell lines, Wnt5a was commonly expressed in primary breast cancer (Fig. 4). Comparing normal breast tissue to tumor tissue showed levels that were 4-fold higher on average in the latter (Table 2). Ten of 28 tumors had levels higher than the highest expression in the cell lines from normal breast tissue. Thus, the cell lines reflected normal breast expression, but not tumor levels. Tumor levels were significantly greater than normal tissue levels (P = 0.0004, Mann-Whitney U test).

Benign breast tissue also showed much higher expression than normal breast tissues, similar to and often greater than levels in many of the tumors. Two different types of benign breast disease were studied: fibroadenomas and fibrocystic disease. The former is a benign lesion involving epithelial and stromal elements. The latter is a nontumorous collection of cysts and ducts with some epithelial hyperplasia. In both types of benign breast disease there was high expression of Wnt5a (Fig. 4, nuclease protection). Benign levels were significantly greater than those of normals (P = 0.0012 Mann-Whitney U test) and also than those of tumors, although to a smaller extent (P = 0.03).

In situ hybridization using the Wnt5a probe was carried out to assess localization of expression. This showed that levels were low and difficult to detect in normal breast but were detectable in a few ducts and lobules. In some larger ducts the mRNA appeared to be expressed in luminal cells but not the myoepithelial population. In fibroadenomas there was high expression in the epithelial component uniformly throughout the tumor (Fig. 5, a and b). In fibrocystic disease it was again
was not due to gene amplification since Southern blots of the breast cancers. Wnt5a probes used as controls for nonspecific hybridization showed an increased localization at the invading edge. The sense clumps of invading cells throughout the stroma. There was no expressed within the epithelial element of the tumor and in breast cancer cell lines. A RNase protection assay on cell lines. Fig. 3 BT2O; m, EGFR (fmol/mg) ER (fmol/mg) Age (yr) c, especially, in the malignant tumors (Fig. 5, e and f) Wnt5a was expressed within the epithelial element of the tumor and in clumps of invading cells throughout the stroma. There was no increased localization at the invading edge. The sense Wnt5a probes used as controls for nonspecific hybridization showed a uniform and low background signal (data not shown).

Thus, in many cases of benign breast proliferation and breast cancers. Wnt5a is overexpressed in the epithelium. This was not due to gene amplification since Southern blots of the high-expressing cases showed no evidence of this (data not shown).

Features of carcinomas known to relate to tumor phenotype were compared with Wnt5a expression. There was no correlation of either ER or EGFR with Wnt5a expression (P = 0.3 and 0.5, respectively, Spearman’s rank correlation coefficient; Table 2), nor was there a correlation with node status (P = 0.1). There was no association with menopausal status, as indicated by age >50 years or <50 years old (Table 2). ER and EGFR were inversely related to each other, as previously reported (Ref. 22; P = 0.005, Spearman’s rank correlation coefficient). ER was related to age and EGFR inversely as has been described before (Ref. 22; P = 0.0007 and 0.036, respectively, Spearman’s rank correlation coefficient).

In relation to patient age, there was no significant difference between normal and benign samples but the primary cancer patients were significantly older than the normal (P = 0.02) and benign (P = 0.0013) samples. However the differences in Wnt5a expression between the samples cannot be a function of the different age distributions, as Spearman’s rank correlation of Wnt5a with age as a continuous variable shows no correlation (P = 0.11). Furthermore the group of tumors which overlap the normal and benign samples in age (n = 9, age <52 years) have no different a level of Wnt5a than those which are older (n = 19, age >52 years, P = 0.86). Also, the differences in the level of Wnt5a between normal samples and cancers are maintained whether the cancer patients considered are over or under 52 years of age. Thus, the significant differences in Wnt5a expression seen between the groups are not related to age distribution differences.

**DISCUSSION**

The human Wnt5a gene shows marked homology to other species including mouse (23) and Xenopus (24). This is characteristic of all of the Wnt family members which are highly conserved (25). They show greater homology to the family member in different species than to other family members expressed in the same species. Wnt5a is located on the terminal region of chromosome 3 beyond the 3p11 band, whereas mouse Wnt5a is on chromosome 14. However, human chromosome 3 is syntenic with mouse chromosome 9, suggesting chromosomal rearrangements at this locus during evolution. The region 3p21-25 is known to be involved in loss of heterozygosity in human cancer (26), including 30% of breast cancers (27). How-

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**Table 2 Wnt5a expression assayed by RNase protection in human breast cancer**

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Benign (n = 10)</th>
<th>Normal Tissue (n = 15)</th>
<th>Primary (n = 28)</th>
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<tbody>
<tr>
<td>Age (yr)</td>
<td>Total</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>49</td>
<td>39</td>
<td>42</td>
</tr>
<tr>
<td>SE</td>
<td>2.13</td>
<td>3.1</td>
<td>4.8</td>
</tr>
<tr>
<td>Median</td>
<td>49</td>
<td>38</td>
<td>46</td>
</tr>
<tr>
<td>Min, max</td>
<td>18, 86</td>
<td>25, 52</td>
<td>18, 78</td>
</tr>
<tr>
<td>Wnt5a</td>
<td>(RNA units)</td>
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<td></td>
</tr>
<tr>
<td>Mean</td>
<td>7.1</td>
<td>18.7</td>
<td>1.5</td>
</tr>
<tr>
<td>SE</td>
<td>10.4</td>
<td>17.2</td>
<td>1.5</td>
</tr>
<tr>
<td>Median</td>
<td>3.0</td>
<td>11.5</td>
<td>1.0</td>
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<td>Min, max</td>
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<td>0.5</td>
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<tr>
<td>ER (fmol/mg)</td>
<td>cytosol protein</td>
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<tr>
<td>Mean</td>
<td>128</td>
<td></td>
<td></td>
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<tr>
<td>SE</td>
<td>168</td>
<td></td>
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</tr>
<tr>
<td>Median</td>
<td>50.5</td>
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</tr>
<tr>
<td>Min, max</td>
<td>3, 695</td>
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<tr>
<td>EGFR (fmol/mg)</td>
<td>membrane protein</td>
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<tr>
<td>Mean</td>
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</tr>
<tr>
<td>SE</td>
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</tr>
<tr>
<td>Median</td>
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<td></td>
</tr>
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</tr>
<tr>
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<td>Negative</td>
<td>15</td>
<td></td>
</tr>
</tbody>
</table>

* Min, minimum; max, maximum.

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expressed in the epithelial component (Fig. 5, c and d). Similarly, in the malignant tumors (Fig. 5, e and f) Wnt5a was expressed within the epithelial element of the tumor and in clumps of invading cells throughout the stroma. There was no increased localization at the invading edge. The sense Wnt5a probes used as controls for nonspecific hybridization showed a uniform and low background signal (data not shown).

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**Fig. 3** RNase protection assay on cell lines. Wnt5a and GAPDH signals are shown. Lanes a–f, human mammary epithelial cell lines; lanes g–s, human breast cancer cell lines. a, 2-5-2a; b, 3-4-1; c, 5-3-1; d, 6-1-1; e, MTSV1-7; f, MRSV4-1; g, MCF10; h, ZR11; i, ZR4; j, MDA415; k, MDA453; l, MDA457; m, BT20; n, SKBR3; o, Adriamycin-resistant; p, MCF7; q, AR9B11; r, T47D; s, MDA231.
I.

Fig. 4 RNase protection assay on human breast tissues. Wnt5a and GAPDH signals are shown for 15 normal breast tissue samples, 10 samples of benign disease, and 21 of the 28 tumors examined (data for remaining 7 tumors not shown in this figure but included in Table 2).

Fig. 5 In situ hybridization of Wnt5a in human breast tissues. a, fibroadenoma, light field; b, fibroadenoma, dark field; c, fibrocystic disease, light field; d, fibrocystic disease, dark field; e, carcinoma, light field; f, carcinoma, dark field. e, epithelial component; s, stroma; c, carcinoma.

ever, the work of Clark et al. (28) suggests that Wnt5a is outside this region and therefore not the gene involved.

During the course of this work, the isolation of overlapping clones was reported giving the total sequence of the human Wnt5a cDNA isolated from a human fetal fibroblast library. We have similarly cloned overlapping clones from a human placental library. Our results independently confirm the sequence published by Clark et al. (28), as well as the chromosomal localization of the gene. Our sequence differs from that of Clark et al. (28) at a few nucleotides but these do not give rise to amino acid differences.

Expression was studied in a range of human breast cancer cell lines representing ER-positive and -negative types as well as those having amplification of EGFR or erbB-2. With the exception of BT20, expression in the cell lines was very low with levels similar to or lower than those found in normal tissues. The breast cancer cell line BT20 showed high wnt5a expression, similar to the elevated levels found in benign proliferative lesions. In the mouse, Wnt1 expression in mammary epithelium produces abnormal morphology in a hormone-independent fashion (29). In relation to this, the level of expression of Wnt5a in breast cancer cell lines appears unrelated to hormone receptor status.

In tissues, Wnt5a expression was generally higher than that in cell lines. Benign and malignant proliferative lesions of the breast respectively showed levels of Wnt5a 10-fold and 4-fold
higher than those in normal tissue. In the mouse Wnt5a is expressed in normal breast tissue and its regulation has been studied over a short period of reproductive history (10). Wnt5a is present in early pregnancy but is undetectable by day 17.5 of pregnancy. It is clearly difficult to reproduce such studies in humans, but our results suggest that human breast tissue generally resembles that of the mouse in its expression of Wnt5a, although no major endocrine effects involving steroid hormones were demonstrated in that pre- and postmenopausal breast levels were no different in any of the patient groups. This does not, however, exclude pregnancy-specific regulation events. Wnt5a was highest in the benign proliferative lesions, and analysis in human breast cancer showed up-regulation of Wnt5a in 10 of 28 carcinomas above the highest level in normal breast tissues. The up-regulation was not due to gene amplification in the breast cancers and may therefore be related to transcriptional activation or RNA stabilization. The elevated level of Wnt5a is independent of several other factors known to affect prognosis or biology of human breast cancer such as EGFR and estrogen receptor. In order to understand further the basis of up-regulation of Wnt5a in human breast disease, human breast cell lines transfected with a panel of oncogenes and cultured in different extracellular matrices have been studied for regulation of Wnt5a. There is evidence that Wnt5a is up-regulated severalfold in these circumstances. Thus, Wnt5a may be secondarily regulated in response to a range of other genetic changes. Since Wnts have recently been shown to modulate cell adhesion by regulation of cadherins, it is possible that Wnt5a may also contribute to this mechanism of regulation of growth and differentiation.

In the mouse, Wnts have a role in the development of normal mammary tissue, and aberrant Wnt expression can contribute to mammary hyperplasia and mammary carcinomas. Drawing a parallel between these observations and human Wnt5a, the expression of the gene in normal breast suggests that it has a role in this tissue, and its aberrant expression is associated with proliferative lesions or aberrant differentiation as in fibroadenomas and fibrocystic disease. Olson and Papkoff (30) have reported that the level of Wnt5a expression is inversely related to the proliferative rate of the mouse mammary epithelial cell line C57MG in vitro. Although this murine result cannot necessarily be extrapolated to the human situation, it argues against Wnt5a having a simple growth promoting role in the benign and malignant lesions we analyzed. Thus, Wnt5a is expressed at its highest level in benign disease, although it is also elevated in cancer. It is possible that high Wnt5a expression occurs at certain stage of normal differentiation, and that cells blocked in this phase show the observed regulation. Olson and Papkoff (30) have shown that in the mouse mammary epithelial cell line C57MG, transfection of Wnt1 and Wnt2 causes a 30-fold reduction in the expression of endogenous Wnt4 and a smaller decrease in the expression of Wnt5a. Thus, it is possible that Wnt5a itself may interfere with the function of other Wnts in human breast tissue where it is overexpressed.

To assess localization of Wnt5a, in situ hybridization was carried out on normal tissues, fibrocystic disease, fibroadenomas, and carcinomas. In normal tissues, the level of expression was generally too low to detect above background, but could be seen in a few ducts. In fibrocystic disease, fibroadenomas, and carcinomas Wnt5a was expressed in epithelium. In some cases it was possible to clearly resolve the basal myoepithelial layer of the breast from the luminal epithelium and Wnt5a was expressed in the luminal cell layer.

In fibroadenomas stromal cells are present in a much greater proportion than in normal epithelium. Dilution of RNA from the epithelium by matrix and stromal cells shows how high the level of RNA must be in the fibroadenoma epithelium compared to normal tissue epithelium. It is possible that the stromal cells are involved in regulation of Wnt5a expression in the epithelium or conversely that Wnt5a expression effects stromal growth. Coculture of luminal cells with stromal cells to measure Wnt5a regulation would be helpful to assess the role of such stromal epithelial interactions.

The up-regulation of Wnt5a in both benign and malignant proliferative disease of the breast suggests an important role of Wnt genes in breast pathology. Wnt5a is the first member of the family to demonstrate up-regulation in human breast cancer. Cell lines established from benign or normal tissues may be suitable models for further evaluation of the role of Wnt5a to assess its regulation under variable growth and differentiation conditions.

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