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Expression of Wnt5a Is Downregulated by Extracellular Matrix and Mutated c-Ha-ras in the Human Mammary Epithelial Cell Line MCF-10A[1]

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Wnt genes are involved in tumour growth and regulate cell adhesion. Some (Wnt5a and Wnt7b) are more highly expressed in human breast cancer compared to normal tissues. Wnt5a is involved in the regulation of cell movement in *Xenopus* and is upregulated in several human cancers. Factors regulating Wnt gene expression in human breast epithelium are poorly understood, but c-erbB2 is amplified in many breast cancers and associated with rapid growth and metastasis, as is high expression of c-Ha-ras. To further understand the regulation of Wnt gene expression, this study investigated the effect of proto-oncogenes c-Ha-ras and c-erbB2, and collagen on Wnt mRNA expression, in a normal spontaneously immortalised human mammary epithelial cell line MCF-10A. Out of nine human Wnt genes investigated, Wnt5a and Wnt7b were expressed in the parental cell line, and neomycin-, c-Ha-ras- and c-erbB2-transfected cell lines. The level of Wnt5a mRNA expression was decreased 40-fold and 3-fold when parental cells were grown on collagen and in collagen, respectively. This downregulation correlated with cell branching. However, Wnt7b was not regulated by collagen. In the presence of activated c-Ha-ras, the level of Wnt5a mRNA expression was markedly decreased (> 200-fold) and cell growth rate was elevated. When treated with $p21^{ras}$ inhibitor, BZA-5B, there was a moderate reversal of Wnt5a mRNA expression (2-fold) with a parallel decrease in cell growth. The data indicate that c-Ha-ras is an upstream inhibitory regulator of Wnt5a, and provide further evidence of an inverse relationship between Wnt5a mRNA expression and cell branching. This demonstrates selectivity of regulation of individual members of the Wnt gene family by the ras pathway. Overexpression of c-erbB2 had no effect on Wnt5a or Wnt7b mRNA expression. Thus, extracellular matrix and ras regulate Wnt5a, providing a mechanism for feedback of morphogenetic movements, which is relevant also to cancer biology.

Key Words: **Wnt5a; Wnt7b; c-Ha-ras; regulation; MCF-10A.**

Wnt genes encode a family of developmentally highly conserved cysteine-rich glycoproteins of ~45 kDa that is present in invertebrates and vertebrates (1). The Wnt proteins are secreted and act in a paracrine and/or autocrine manner, like growth factors, to elicit biological responses associated with normal developmental processes as well as cell proliferation and differentiation (1,2). The mechanism of action of Wnts involves signalling via frizzled and dishevelled. *In vitro* murine Wnt1 has been shown to regulate β-catenin pool size and stabilise β-catenin/cadherin complexes, which are also known to be associated with tumour cell invasion (3-6). Wnt1 causes partial transformation of mammalian epithelial cell lines (7), affects the cell morphology in the mouse embryonic fibroblast cell line C3H 10T1/2 (8) and alters the cell morphology induced by growth factors via the MAP kinase pathway in the rat pheochromocytoma cell line, PC12 (9). In *Xenopus laevis*, Wnts are involved in the normal axis formation and their selectivity resides in the C-terminal regions (10). Wnts act co-operatively with basic fibroblast growth factor (bFGF) to determine embryonic pattern (11). *In vivo* the introduction of mouse mammary epithilia producing Wnt1 into reconstituted mammary gland induces hyperplasia that is hormonally independent

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(12). The same phenotypic change has also been seen in mouse mammary tumour virus (MMTV)-induced Wnt1 activation in C3H mouse mammary gland which subsequently leads to adenocarcinoma after a latent period (13), and in Wnt1 transgenic mice (14). The rate of Wnt1-induced mammary hyperplasia is increased in Wnt1/FGF3 bitransgenic mice indicating a co-operative role of Wnt and FGF in the murine breast tumourigenesis (15).

In humans, the Wnt5a mRNA level is increased in breast, colon, lung and prostate carcinomas and melanoma versus normal tissues (16,17), Wnt7b is increased in breast cancer (18), Wnt2 in colon carcinoma and breast fibroadenoma and Wnt4 in breast fibroadenoma (18,19). Altogether the data suggest the role of some Wnt genes in the progression of human malignancy. Several factors have been reported to down-regulate Wnt4 and Wnt5a gene expression *in vitro* including bFGF protein, *int-2* gene, Wnt1 gene, Wnt2 gene and mutated *neu* tyrosine kinase receptor gene transfection. Furthermore, the down-regulation of Wnt4 and Wnt5a is inversely related to cell proliferation (20). Disruption of the cytoskeleton or treatment with hepatocyte growth factor can also down-regulate Wnt5a mRNA level in the immortalised human mammary epithelial cell line, HB2. This Wnt5a down-regulation is associated with cell branching. At confluence, Wnt5a mRNA level is increased (21). In *Xenopus laevis*, Wnt5a has been shown to block the elongation of blastula caps (22). The data suggest that *in vitro* Wnt5a acts as a modulator of cell migration and its gene expression is regulated by multiple factors.

Therefore to extrapolate from these data, the level of Wnt5a mRNA would be expected to be low in cancer tissues *in vivo* compared to corresponding normal tissues. However, the opposite has been reported in several human cancers (16-19). The discrepancy of Wnt5a mRNA expression level between *in vivo* and *in vitro* suggests the need to understand further the factors that regulate Wnt5a mRNA expression and the pathways controlling the basal level of Wnt5a mRNA. We studied the spontaneously immortalised human breast epithelial cell line, MCF-10A, which represents the normal human luminal mammary epithelial cells (23), the effects of extracellular matrix and oncogenes known to be involved in human breast cancer either via gene amplification (erb-B2) or high expression (H-ras).

MATERIALS AND METHODS

Materials. Parental MCF-10A cell line (MCF-10Awt), and its derivatives containing the neomycin-resistant gene (MCF-10Aneo) or the human T24-mutated c-Ha-ras oncogene (MCF-10AneoT) have been described (24). MCF-10Aneo and MCF-10AneoT cells contained a pool of 30 isolated colonies each and were used in the study. MCF-10A c-erbB2 cell line has been established previously (25). BZA-5B compound (mw = 571 g/mol) was obtained from Genentech Inc., South San Francisco, CA 94080, USA and was prepared by dissolving in dimethylsulfoxide (DMSO) / dithiothreitol (DTT). All laboratory reagents were from Sigma unless otherwise stated.

Cell culture and RNA preparation. MCF-10A cells were grown in Dulbecco's modified Eagle's medium (DMEM) / Ham's F-12 (1:1) medium (Imperial Cancer Research Fund Clare Hall Laboratories, U.K.) supplemented with 10% fetal calf serum (Globepharm), 20 μg/ml insulin, 0.5 μg/ml hydrocortisone and 10 ng/ml epidermal growth factor (24). Total RNA was prepared from cells using the acid quanidium thiocyanate-phenol-chloroform extraction method (26), followed by a 5.7 M caesium chloride separation in polyallomer tubes (13 × 51 mm, Beckman) at 50 K rpm for 3 hours using SW50 or SW55 swing rotor (Beckman) in the L8-80M ultracentrifuge (Beckman). The RNA pellet was resuspended in 200 μl of sterile water, treated with RNase-free DNase at 37°C for 15 minutes, extracted with an equal volume of phenol, ethanol precipitated with 0.1× volume of sodium acetate pH 5.2 and resuspended in water to the final volume of 1 μg/μl.

Cell culture in the presence of collagen. Cells were grown on collagen by spreading out 3 ml per 10 cm diameter culture plate of collagen prepared as instructed by the manufacturer (Invitrogen), allowed to set at room temperature for 30 min and then 10 ml of resuspended cells in culture medium were poured gently on top. For cells growing in collagen, 0.3 ml of resuspended cells in culture medium were mixed with 3 ml of collagen, and then poured gently onto a 10 cm diameter culture plate. Collagen was allowed to set in a 37°C humidified incubator for 30 min and then 10 ml of culture medium was poured gently on top. Cells were plated out at 100% confluence, fed after 2 days and harvested 4 days post-plating. For cells grown on collagen or in collagen, 5 ml of collagenase 1 mg/ml was added to the plate to digest collagen to completion in the incubator prior to harvesting.

Subconfluence and confluence growth, and treatment with BZA-5B. In the subconfluence and confluence experiments, MCF-10A cells were plated out at an equal density to about 50% confluence and allowed to grow until ~80% confluence (subconfluence) or 100% confluence. The BZA-5B treatment experiment was carried out in triplicate on 10-cm plates. Again, MCF-10A cells were plated out at an equal density to about 50% confluence to which the following was added: 50 mM BZA-5B, DMSO/DTT (solvent control) or culture medium alone. Every 3 days, the medium was replaced with fresh medium containing an appropriate treatment. Total RNA was then harvested at day 5.

Treatment with BZA-5B and cell count. MCF-10A cells were plated out in triplicate at a density of 300 cells per well in the 24-well plate. The cells were either untreated or treated with solvent alone or 50 mM BZA-5B. A fresh medium was replaced every three days. Every 24 hours post-plating, 3 wells from either MCF-10Awt, MCF-10Aneo or MCF-10AneoT cell lines were trypsinised in 0.5ml volume, and transferred to 9.5ml Isoton II (Coulter). The cell number was counted using the coulter counter ZM (Coulter) from which the absolute read out was recorded.

Ribonuclease (RNase) protection analysis. The human Wnt5a (21), Wnt10b (27) and Wnt 2, 3, 3a, 4, 7b and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (18) have been described. The linearised Wnt and GAPDH plasmid DNAs were labelled with [α-^{32}P]CTP to generate antisense riboprobes which were then purified using the spin column according to the manufacturer's instructions (Boehringer Mannheim). RNase protection analysis was performed on 10 μg of total RNA at 45°C using standard protocols (28). Autoradiography was done at −70°C with intensifying screens. Yeast transfer RNA (Boehringer Mannheim) was used as a negative control. The protected fragment signals for Wnt and GAPDH were quantified by laser densitometry using a Bio Image analyser (Millipore). GAPDH was used as an internal loading control.

TABLE 1

Wnt mRNA Expression in MCF-10A Wild Type and Transfectants

Wnt	2	3	3a	4	5a	7a	7b	10b
MCF-10Awt	−	−	−	−	++	−	+	−
MCF-10Aneo	−	−	−	−	++	−	+	−
MCF-10AneoT	−	−	−	−	+	−	+	−
MCF-10A c-erbB2	−	−	−	−	++	−	+	−

++ = high expression, + = low expression, − = no expression.

Statistical analysis. The level of Wnt mRNA expression was calculated as a ratio of Wnt/GAPDH protected fragment signals. The level of expression of Wnt5a in BZA-5B treated and untreated cells was compared using Student's unpaired t-test from the Minitab version 8.2.

RESULTS

Wnt mRNA expression in MCF-10A wild type and transfectants. Table 1 summarises the Wnt mRNA expression in MCF-10Awt, MCF-10Aneo, MCF-10AneoT and MCF-10A c-erbB2 cell lines. The constitutive expression of mutated c-Ha-ras in MCF-10AneoT cells resulted in a significant down-regulation (> 200-fold) of Wnt5a mRNA level compared to MCF-10Awt and MCF-10Aneo (Fig. 1). Overexpression of c-erbB2 had no effect on Wnt5a mRNA expression compared to MCF-10Awt and MCF-10Aneo. The level of Wnt7b mRNA was constant in all MCF-10A cell lines. Wnt 2, 3, 3a, 4, 7a and 10b mRNAs were not detected in any MCF-10A cell line. The results were obtained from cells growing to confluence on plastic surface, and showed that mutated c-Ha-ras down-regulates Wnt5a mRNA expression.

Effects of Wnt5a and Wnt7b mRNA expression in the presence of collagen. One pathway by which the above effects may be mediated is signalling from the extracellular matrix and changes in cell adhesion which characteristically occur in transformed cells and cancer. Therefore, the effect of collagen on Wnt5a and Wnt7b mRNA expression was investigated. The cells were plated out at a confluence density and allowed to grow for four days prior to harvest. There was a 40-fold and 3-fold down-regulation of Wnt5a mRNA level in MCF-10Aneo cells grown on collagen and in collagen, respectively, compared to on plastic (Fig. 2, C). The Wnt5a down-regulation was correlated with the appearance of increased cell branching (Fig. 2, A). In the MCF-10AneoT cells, the level of Wnt5a mRNA was also lower in cells grown on collagen compared to on plastic, and was approximately equal in cells grown in collagen compared to growth on plastic. This was difficult to quantify since the intensity of Wnt5a protected fragment was very low in MCF-10AneoT cells (Fig. 2, C). Fig. 2, B shows the change of morphology when MCF-10AneoT cells were grown in 3 different conditions. Collagen had no effect on the level of Wnt7b mRNA expression in or between MCF-10Aneo and MCF-10AneoT on plastic, on collagen or in collagen (data not shown). Therefore, collagen down-regulated Wnt5a mRNA expression selectively.

Wnt5a mRNA expression is not regulated by confluence. The effect of cell density on Wnt5a mRNA expression was investigated since it was previously reported that confluence upregulates Wnt5a mRNA level in HB2 cells (21). The cells were plated out on plastic at a subconfluence density and were harvested at the following times: 0.5, 1, 2, 3 and 5 days. There was no change in Wnt5a mRNA level throughout the time course in MCF-10Aneo or MCF-10AneoT (data not shown). Therefore, confluence had no effect on Wnt5a mRNA expression.

Reversal of Wnt5a mRNA down-regulation in MCF-10AneoT cell line by BZA-5B. BZA-5B, a farnesyl protein transferase inhibitor that has been shown to block specifically the synthesis of biological active $p21^{ras}$ protein and subsequently reversed the abnormal cell growth induced by activated H-ras(Gly12Val) (29). Fig. 3, B shows that BZA-5B inhibited the abnormal cell growth in MCF-10AneoT cells at day 4 by ~45% compared to no treatment and by ~56% at day 7. The reduced cell growth led to a sparse cell population in BZA-5B treated MCF-10AneoT cells compared to untreated (data not shown). This was associated with a moderate increase in the Wnt5a mRNA level (mean = 2-fold) in BZA-5B treated MCF-10AneoT cells

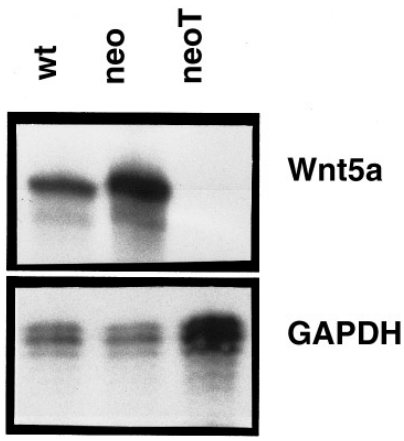

FIG. 1. RNase protection analysis showing the downregulation of Wnt5a mRNA expression in MCF-10AneoT. wt = MCF-10Awt, neo = MCF-10Aneo and neoT = MCF-10AneoT. 20 μg of total RNA was used in neoT whereas 10 μg was used in wt and neo. Total RNA was incubated with antisense Wnt5a riboprobe at 45°C, digested with RNaseA, separated onto a 6% polyacrylamide/urea gel and autoradiographed.

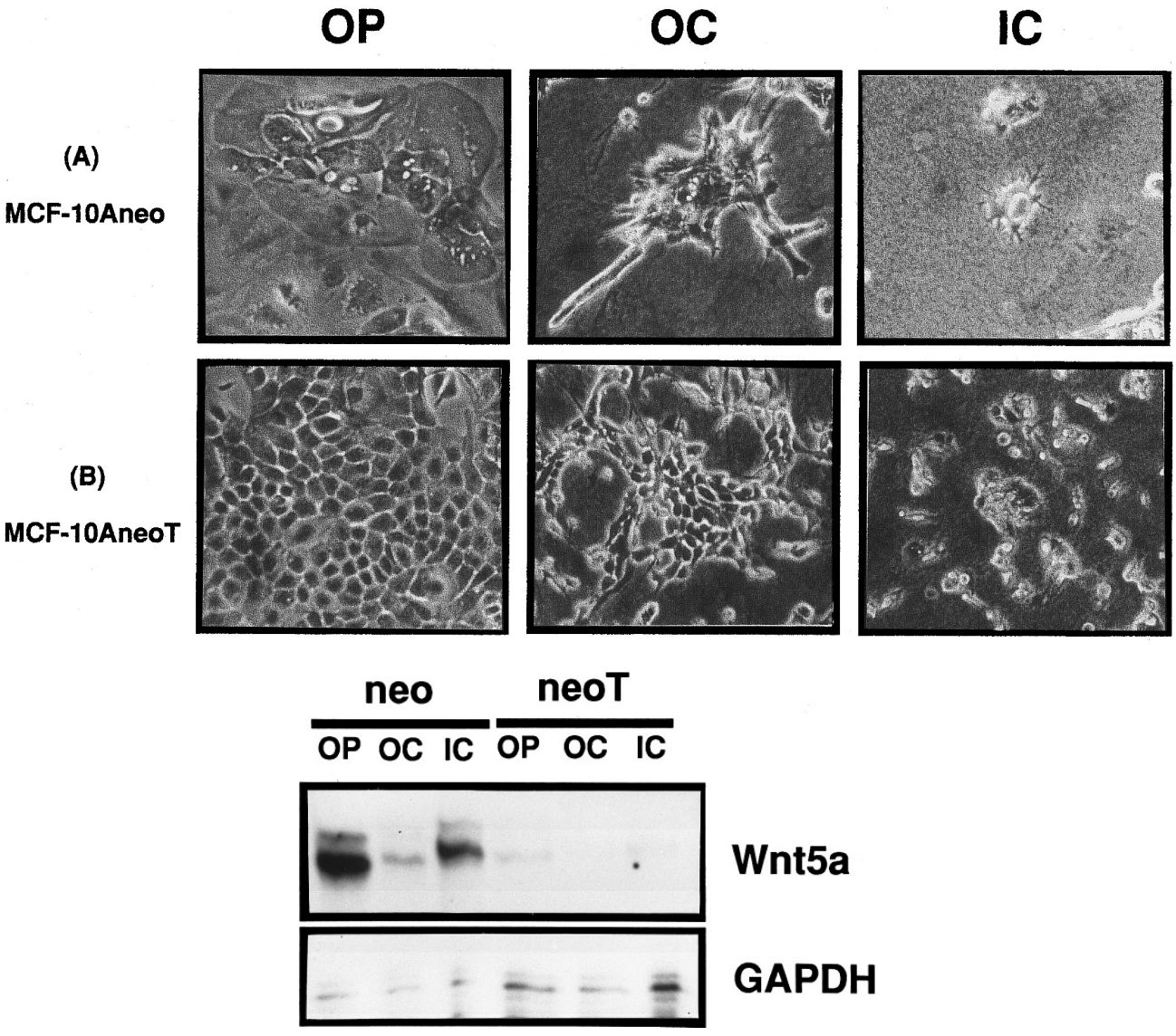

FIG. 2. Effect of MCF-10A in the presence of collagen. The morphology of MCF-10Aneo (A) and MCF-10AneoT (B) cells in different culture conditions: OP = on plastic, OC = on collagen and IC = in collagen. Magnification 100×. (C) RNase protection analysis showing the corresponding level of Wnt5a mRNA. The level of Wnt5a mRNA was expected to be low in neoT (see Fig. 1). neo = MCF-10Aneo and neoT = MCF-10AneoT.

compared to untreated (P = 0.001) (Fig. 3, C & E). This experiment was performed in triplicate and repeated twice in which a similar degree of reversal of Wnt5a mRNA level was seen in MCF-10AneoT treated cells. BZA-5B had no effect on cell growth inhibition and Wnt5a mRNA level in MCF-10Aneo cells (Fig. 3, A & D). Therefore, inhibition of the sysnthesis of biological active $p21^{ras}$ protein led to a moderate reversal of Wnt5a mRNA level in MCF-10AneoT but not MCF-10Aneo cells.

DISCUSSION

Wnts are a group of novel growth factors that play a role in mouse tumourigenesis and possibly in the progression of human malignancy. The mechanism of their action is not yet fully understood, although *in vitro* Wnt1 has been demonstrated to act via cell adhesion molecules (5,6) and to increase cell proliferation (20). Recently we have used a normal human mammary epithelial cell line, HB2, which has been immortalised with the T antigen of SV40, to demonstrate the association of Wnt5a gene down-regulation with cell branching (21). In this study, we examined a normal spontaneously immortalised human mammary epithelial cell line, MCF-10A (23), and showed that the down-regulation of Wnt5a mRNA level is also associated with cell branching. This occurred when cells were grown in the presence of collagen suggesting

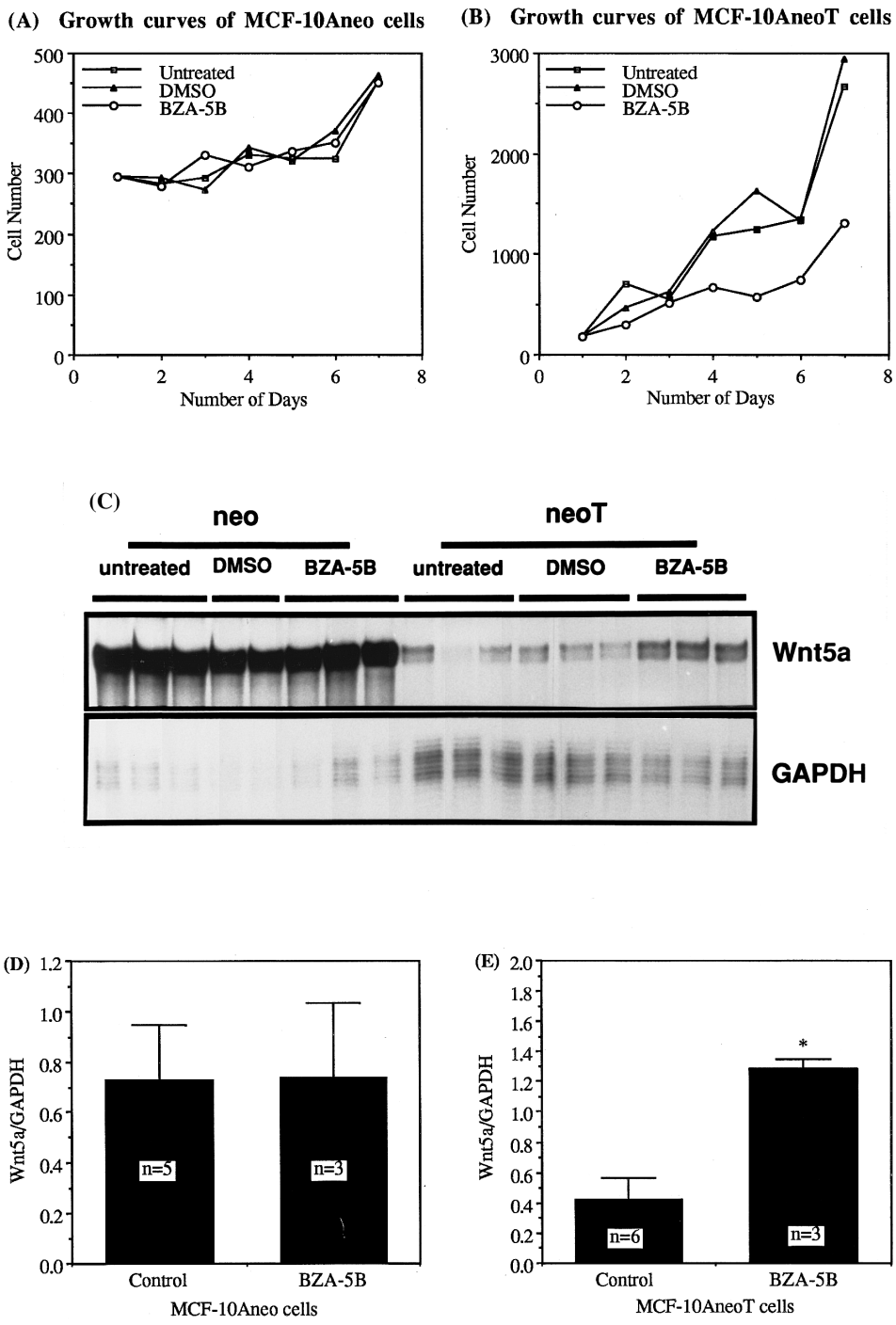

FIG. 3. Effect of BZA-5B on MCF-10A cell growth and Wnt5a mRNA expression. Cell growth of MCF-10Aneo (A) and MCF-10AneoT (B), and the corresponding Wnt5a mRNA expression on treatment with 50 μM of BZA-5B (C) detected by RNase protection analysis (see Fig. 1). The level of Wnt5a mRNA expression was quantified and represented in (D) for MCF-10Aneo and (E) for MCF-10AneoT. control = pooled samples of untreated and DMSO, * = significant difference. 20 μg total RNA was used in all MCF-10AneoT cells compared to 10 μg in MCF-10Aneo to enhance the density of Wnt5a protected fragment.

that there is an association of extracellular matrix (ECM) signalling and the down-regulation of Wnt5a in the breast epithelial cells.

Parental MCF-10A cells expressed only Wnt5a and Wnt7b genes of the Wnt genes analysed, both of which have been reported to be elevated in human breast

cancers (17,18). In this study, activated c-erbB2 had no effect on Wnt5a mRNA expression. However, it was shown that transfection of activated *neu* tyrosine kinase receptor gene down-regulated Wnt5a mRNA expression in a mouse breast cancer cell line (20). Therefore, this contrasting result highlights the necessity to study normal breast epithelial cell lines to understand relevance of these Wnt genes to human breast cancer.

From our data, stably transfected mutant c-Ha-ras proto-oncogene significantly down-regulated the level of endogenous Wnt5a mRNA but had no effect on the level of endogenous Wnt7b mRNA in MCF-10A, suggesting two distinct regulatory pathways for Wnt5a and Wnt7b in the MCF-10A cell model, and that c-Ha-ras is an upstream regulator of Wnt5a mRNA expression. In contrast to C57MG, the down-regulation of Wnt5a in MCF-10AneoT was unlikely to be caused by cell proliferation for two reasons: (i) the level of Wnt5a mRNA was unchanged from low density to high density cultures, and (ii) the level of Wnt5a mRNA was unchanged between MCF-10A c-erbB2 transfected cells and wild type. MCF-10A c-erbB2 transfected and MCF-10AneoT cells have a higher growth rate than wild type cells (30).

To further assess the role of mutant c-Ha-ras, BZA-5B compound was used to block the specific synthesis of mutant $p21^{ras}$ protein in MCF-10AneoT and a moderate increase in Wnt5a mRNA level was detected. This indicates that mutant c-Ha-ras can down-regulate Wnt5a mRNA expression. The moderate increase in Wnt5a mRNA level seen after BZA-5B treatment may be due to incomplete inhibition of all mutant $p21^{ras}$ protein synthesis. Also, Wnt5a mRNA expression might be more sensitive than growth inhibition to mutant $p21^{ras}$ protein.

In vivo, the level of Wnt5a mRNA has been reported to be elevated in breast cancer but the mechanism of regulation is unknown (16,17). *In vitro,* the HB2 cell line also exhibited elevated Wnt5a mRNA level at confluence (21). However, this Wnt5a gene upregulation was not detected in the MCF-10A cell line suggesting that the difference could be caused by the mechanism of immortalisation of HB2 which might alter the genetic components regulating Wnt5a.

There is an emerging evidence showed that ras may be involved in the ECM signalling via integrins to regulate gene expression (31 and references therein). This is in agreement with our finding here. We showed that ECM is associated with Wnt5a gene down-regulation and that mutated c-Ha-ras can also cause Wnt5a gene down-regulation. Thus in the MCF-10A cell model, ras can mimick the ECM signalling to down-regulate Wnt5a gene expression, and so provides a link between ECM signalling, ras expression and regulation of Wnt5a gene. This result may be important to cell migration, a role that has been implicated for Wnt5a (21,22). However, our result here still cannot explain the marked upregulation of Wnt5a in human cancers (16,17). Therefore, this suggests that there must be other mechanisms upregulating Wnt5a in cancer.

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