Repression of Wnt-5a impairs DDR1 phosphorylation and modifies adhesion and migration of mammary cells

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Accepted 15 March 2001
Journal of Cell Science 114, 2043-2053 (2001) © The Company of Biologists Ltd

SUMMARY

The *Wnt-5a* gene encodes a secreted protein that controls several normal processes during embryogenesis and development of adult tissues by as yet unknown mechanisms. Endogenous expression of Wnt-5a mRNA is known to occur in both mouse and human mammary cell lines. To investigate the biological role of Wnt-5a in the human mammary epithelial cell line HB2, we used an antisense approach to repress endogenous expression of Wnt-5a protein. We also generated a cell population that constitutively overexpresses this protein. We found that overexpression of Wnt-5a protein enhanced cell-to-collagen binding and abolished hepatocyte growth factor-stimulated migration of HB2 transfectants through collagen matrices. Conversely, repression of Wnt-5a protein led to cell scattering, impaired cell-collagen interaction and enhanced cell motility. As we were searching for modified collagen receptors in antisense cells, we discovered that the collagen-binding discoidin domain receptor 1 (DDR1) failed to undergo phosphorylation. In reciprocal experiments, phosphorylation of DDR1 was consistently enabled by expression of Wnt-5a-HA protein in non-Wnt-5a-producing MCF-7 breast cancer cells. Activation of the Wnt/β-catenin signalling pathway did not influence or mimic the Wnt-5a-mediated effect on DDR1 phosphorylation. These data demonstrate that Wnt-5a protein participates in regulation of adhesion to and migration through collagen and is also a co-factor necessary for collagen-induced activation of DDR1 receptors in mammary epithelial cells.

Key words: Adhesion /DDR1 phosphorylation/Mammary epithelial cells/Migration/Wnt-5a

INTRODUCTION

Members of the *Wnt* gene family encode signalling glycoproteins that are involved in embryogenesis and in the regulation of a wide variety of normal and pathological processes (for reviews, see Moon et al., 1997; Cadigan and Nusse, 1997; Dale, 1998). Wnt factors are secreted from the cells and bind to the outer surface of the plasma membrane or to the extracellular matrix (ECM; Papkoff et al., 1987), and from there they interact with their receptors, designated Frizzled (Fz; Yang-Snyder et al., 1996; Wang et al., 1996), to initiate diverse intracellular signalling. The repertoire of Fz receptors and other unknown components present on the cell surface or in the ECM determines what type of intracellular signalling pathway will be inaugurated. To facilitate discrimination of the different Wnt signalling pathways, Miller et al. (1999) have proposed a division into two main pathways, referred to as the Wnt/β-catenin and the Wnt/Ca^{2+} pathway, to emphasise the downstream intracellular effectors.

Both of the mentioned signalling pathways can be activated by Wnt-5a, which is a non-transforming member of the Wnt gene family (Wong et al., 1994). In the Wnt/β-catenin pathway, the Wnt-5a protein in *Xenopus* embryos exerts its effect through a specific cell surface receptor, Fz-5, and an intracellular signalling pathway that leads to activation of the β-catenin pathway and thereby to axis duplication (He et al., 1997). It has been shown for the Wnt/Ca^{2+} pathway that ectopic expression of XWnt-5a in zebra fish embryos stimulates the phosphatidyl inositol signalling pathway and enhances the release of intracellular Ca^{2+} (Slusarski et al., 1997).

Co-ordination of the Wnt-5a signal with other internal signals has not been elucidated in humans. However, it has been shown that intracellular components of the Wnt signalling pathways in various species have been highly conserved during evolution (Ramakrishna and Brown, 1993), hence studies of Wnts in other organisms have provided information that might apply to humans as well. For example, it has been reported that the expression of Wnt-5a mRNA is induced during several phases of the development and modulation of mouse breast tissue. This indicates a role for this gene in controlling yet unidentified processes in postnatal breast tissue (Weber-Hall et al., 1994; Gavin and McMahon, 1992). The *Wnt-5a* gene could possibly play the same role in humans, because endogenous expression of Wnt-5a mRNA is known to occur in both mouse and human mammary cell lines and human and mouse Wnt-5a protein exhibits 90% homology at the amino acid level.

To explore the role of Wnt-5a in human breast cells, we chose to use a breast epithelial cell line, HB2, which expresses Wnt-5a endogenously. Consequently, this cell line represents a

suitable model for investigating the mammary epithelial responses to overexpression/repression of Wnt-5a protein. Moreover, growing HB2 cells in three-dimensional collagen matrices induces morphogenesis (Berdichevsky et al., 1994), during which cells are rearranged into three-dimensional structures mimicking the events that happen in vivo. These properties of HB2 cells make it possible to determine whether overexpression/repression of the Wnt-5a protein could influence the mechanisms involved in the formation of these structures.

In the present study, the manipulation of Wnt-5a protein level was most prominently associated with alterations in adhesion and the migratory capacity of HB2 cells. We assume that these physiological changes are linked to biochemical signalling, because repression of the Wnt-5a protein blocked tyrosine phosphorylation/activation of DDR1. The DDR1 and DDR2 receptors constitute a new class of receptor tyrosine kinases (RTKs; for review, see Vogel, 1999) for which several types of collagen have been identified as ligands (Vogel et al., 1997).

To confirm the association between Wnt-5a protein and DDR1 phosphorylation, we performed complementary experiments using the MCF-7 breast cancer cell line, which has retained several characteristics of differentiated mammary epithelium, but has lost the ability to express Wnt-5a mRNA (Lejeune et al., 1995).

MATERIALS AND METHODS

Generation of Wnt-5a antibody

A Wnt-5a polyclonal antiserum was generated in rabbits immunised with a synthesised peptide corresponding to the C-terminal region of human and mouse Wnt-5a protein. The antiserum obtained was purified in two steps. First, all IgGs in the antiserum were collected using a protein-A Sepharose column; thereafter, the eluted fraction was further purified on a Sepharose 4B column (Amersham Pharmacia Biotech, Uppsala, Sweden) containing the immobilised peptide used for immunisation.

The anti-Wnt-5a antibody was used to screen several cell lines for expression of the Wnt-5a protein. NIH 3T3 cells proved to be negative, whereas HB2 and C57MG cells were positive for expression of the Wnt-5a protein, which is consistent with previous reports of Wnt-5a expression at the messenger RNA level (Olson and Papkoff, 1994; Jönsson et al., 1998). The Wnt-5a antibody was also able to detect varying amounts of Wnt-5a protein in isolated Wnt-5a antisense transfectants, providing additional evidence of the specificity of this antibody.

To further confirm the antibody-antigen specificity, we performed peptide-blocking experiments in which the anti-Wnt-5a antibody was incubated with various amounts of synthesised peptide against which the antibody was developed. In subsequent western blot analysis, the intensity of the Wnt-5a signal declined with an increasing peptide-to-antibody ratio and was eventually undetectable at a tenfold excess of the peptide.

Wnt-5a cDNA constructs

The C-terminal end of the Wnt-5a cDNA was tagged with a HA (haemagglutinin) epitope and cloned into plasmid Bluescript KS+, as previously described by Shimizu et al. (1997); this vector was kindly provided by J. Kitajewski (Columbia University, New York, NY, USA). The Wnt-5a-HA cDNA was removed from pBluescript after digestion with *Eco*RI and *Bam*HI restriction enzymes and subcloned into a retroviral vector, pLNCX (Miller and Rosman, 1989), which was a gift from D. Miller (FHCRC, WA, USA). In the pLNCX vector, neomycin phosphotransferase gene expression is controlled by the murine leukaemia virus long terminal repeat, and Wnt-5a-HA cDNA transcription is controlled by an internal CMV enhancer/promoter. To construct a Wnt-5a antisense-expressing vector, we subcloned the full-length Wnt-5a cDNA in antisense orientation (3′-5′) into the pLNCX vector.

Cell lines and transfection

The HB2 cell line (a subclone of the MTSV1-7 line; Bartek et al., 1991) originated from J. Taylor-Papadimitriou's laboratory (ICRF, UK), and was cultured as described by Huguet et al. (1995). The MCF-7, C57MG and NTN 313 cells were cultured according to the recommendation of the American Type Culture Collection (Rockville, MA, USA).

We also used a *Drosophila* S2 cell line transfected with a construct containing temperature-sensitive wingless (Wg) cDNA under the control of the hsp70 promoter (S2HS-Wg), as well as a non-transfected S2 cell line; both lines were gifts from R. Nusse (Stanford University, CA, USA). These cells were cultured at 25°C in Schneider's *Drosophila* medium supplemented with 10% fetal bovine serum.

HB2 cells were transfected with the constructed vectors described above using the calcium phosphate transfection method. Various populations of HB2 cells expressing either the pLNCX-empty vector (control), the pLNCX Wnt-5a-HA (Wnt-5a-overexpressing), or the pLNCX-Wnt-5a-HA vector in antisense orientation (Wnt-5a antisense) were obtained by growing the cells in the presence of 500 μg/ml geneticin (G418; GibcoBRL, MA, USA) and isolating the stable transfectants.

An anti-HA antibody was used to detect Wnt-5a-HA fusion protein in the isolated G418-resistant Wnt-5a-overexpressing transfectants. To exclude any possible phenotype change between the isolated transfectants, we grew them in collagen and compared them with the parental HB2 cells regarding the ability to form compact, spherical phenotypes in collagen gels. Eight of the ten Wnt-5a-overexpressing transfectants exhibited a phenotype identical to that of non-transfected parental HB2 cells. All ten isolated control transfectants displayed a phenotype similar to that of the parental HB2 cells.

To quantify the total amount of Wnt-5a protein (i.e. both endogenous and overexpressed protein) in Wnt-5a-overexpressing cells, we used the anti-Wnt-5a antibody, which recognises both endogenous and overexpressed Wnt-5a protein. Overexpression of Wnt-5a-HA protein led to a 1.5-fold decrease in the endogenous level of Wnt-5a protein, suggesting the involvement of an autoregulatory mechanism. Densitometric analysis revealed a threefold total elevation of this protein in Wnt-5a-overexpressing cells.

The anti-Wnt-5a antibody was used to screen the isolated G418-resistant Wnt-5a antisense transfectants for the degree of repression of endogenous Wnt-5a protein. We detected five transfectants that exhibited a low level of Wnt-5a protein. Three of these transfectants (designated #1, #9, and #12) were selected for further analysis, because they contained only 10-20% of the endogenous Wnt-5a protein found in non-transfected parental HB2 cells. As these transfectants exhibited identical morphology and growth rate, we performed our experiments on transfectants #1 as well as on pools of isolated transfectants.

We also transfected MCF-7 breast cancer cells and isolated 18 G418-resistant transfectants expressing Wnt-5a-HA (Wnt-5a-expressing) or empty vector (control). When performing experiments on cancer cells, transfection and selection procedures can give rise to clonal variability. Therefore, we carried out experiments on three randomly chosen transfectants and controls, as well as on pools of all isolated transfectants.

Cell growth in collagen gels

The different cell populations were harvested by trypsinisation and

washed twice in PBS. Single-cell suspensions were prepared by syringing cells through a 23-G needle. Aliquots of the cell suspension were mixed with a solution of neutralised bovine dermal collagen type I (Vitrogen-100, Collagen Corp, CA, USA) at a 1:10 ratio to give a final concentration of 1×10^5 cells/ml. The cell-collagen mixtures were added to tissue culture plates (6 cm in diameter) and polymerised at 37°C for 1 hour. The cells were allowed to grow within the collagen gel for 7-8 days, and the growth medium was replaced with fresh medium every other day. Thereafter, the cells were incubated in a standard growth medium in the absence or presence of 10 ng/ml recombinant human hepatocyte growth factor (HGF; R&D Systems, European Ltd, UK). According to previous reports, HGF promotes branch formation in HB2 cells when they are grown in collagen gels (Berdichevsky et al., 1994). The appearance of any developing morphogenetic phenotype was documented with a Nikon F 301 camera connected to a Nikon TMS inverted microscope.

Alford et al. have previously described morphogenetic phenotypes produced by HB2 cells as ball-like, cyst-shaped or branching (Alford et al., 1998). Ball-like refers to a compact, spherical structure composed of a central body that lacks protrusions; parental HB2 cells predominantly form ball-like phenotypes in the absence of HGF. However, in the presence of HGF, the ball-like phenotypes develop processes extending from the central body and are therefore called cysts or branching phenotypes. A cyst has by definition fewer than five processes, all of which are shorter than the diameter of the central body, whereas a branching structure has at least five processes, at least one of which is longer than the diameter of the central body. To quantify these phenotypes, we examined and defined at least 100 morphogenetic structures in each of the triplicate plates. The number of each phenotype was expressed as a percentage of the total number of structures present in the gel, and the mean values were calculated.

Cell-to-matrix binding experiments

Sixteen hours before the experiments, the cultured cells were harvested and re-plated. Thereafter semi-confluent cells were detached from the tissue culture plates using cell scrapers, and single-cell suspensions were prepared and washed twice in a serum-free medium. To allow re-exposure of cell surface receptors, the cells were placed in a serum-free medium containing 0.1% BSA and 1.25 mM Mg^{2+} and then incubated at 37°C for 1 hour on a rotator. Single-cell suspensions (1 ml aliquot containing 5×10^3 cells) were plated on 24-well plates coated with collagen type I at concentrations ranging from 1 to 100 μg/ml. The cells were incubated for 2 hours at 37°C after which non-adherent cells were removed from the wells. Attached cells were fixed with 1% glutaraldehyde and stained with crystal violet (0.1% in H_2O). The wells were washed and the relative number of attached cells in each well was evaluated by measuring absorbance at 540 nm in an ELISA Microplate Reader. The mean values for each concentration and population were calculated, and from those values we subtracted nonspecific cell adhesion measured in separate BSA-coated wells.

Immunoblotting and immunoprecipitation

To detect Wnt-5a-HA fusion proteins, 50 μg of total protein from each sample was separated on 12% SDS-polyacrylamide gel, and electrophoretically transferred onto nitrocellulose membranes (Bio-Rad laboratories, CA, USA), which were incubated with a 1:2000 dilution of an anti-HA antibody (Berkeley Antibody Co., CA, USA) for 1 hour at room temperature. To detect the endogenous Wnt-5a protein levels in the cells, the Wnt-5a antibody was diluted 1:2000 and used under conditions similar to those described above.

To investigate the expression and phosphorylation of DDR1 receptors, a collagen type I solution was neutralised and allowed to polymerise at 37°C for 1 hour, in accordance with the manufacturer's instructions. Semi-confluent cells that had been plated 16 hours prior to the experiments were detached from the plates, and single-cell suspensions were prepared and plated on the polymerised collagen gels or on tissue culture plates. They were then incubated for 0-16 hours in time-course studies or only 16 hours in other experiments. The cells were then washed three times and lysed. The resulting lysates were clarified by centrifugation at 10,000 ***g*** for 15 minutes at 4°C, and the protein content in each sample was determined using Coomassie protein assay reagents (Pierce Chemical Co., IL, USA). For immunoprecipitation, samples of the lysates containing 1 mg/ml total protein were pre-cleared with protein-A sepharose alone and subsequently incubated with 3 μg of anti-DDR1 (Santa Cruz Biotechnology, CA, USA) or anti-phosphotyrosine antibody (4G10, Upstate Biotechnology Inc., NY, USA). After separation of the precipitated proteins on SDS-PAGE, the specific antibodies mentioned above were used to detect DDR1 and tyrosine phosphorylated proteins.

Biotinylation of cell-surface integrins was carried out as reported elsewhere (Rosen et al., 1992), using a sulfo-NHS-LC biotinylation kit (Pierce Chemical Co.). Immunoprecipitations were performed as described above, but with an anti-β1 integrin monoclonal antibody (CAM Folio, Becton Dickinson, CA, USA). Biotinylated β1 integrins were detected using horseradish peroxidase-conjugated avidin solution (Pierce Chemical Co.). The membranes were then stripped twice and sequentially reprobed with α2 and α3 anti-integrin antibodies (CAM Folio, Becton Dickinson, CA, USA).

For detection of focal adhesion kinase (FAK) phosphorylation, samples containing 1 mg/ml pre-cleared protein were incubated with 3 μg of anti-FAK antibody (Upstate Biotechnology, CA, USA), and immunoprecipitates were collected as described above. The anti-phosphotyrosine antibody G410 was used for detection of FAK phosphorylation.

Production of conditioned medium

Schneider's medium conditioned by S2HS-Wg or S2 control cells was produced as previously described (van Leeuwen et al., 1994). In short, S2 and S2HS-Wg cells (approximately $1\text{-}2\times10^6$ cells in both cases) were heat shocked by increasing the temperature to 37°C for 30 minutes and were subsequently allowed to recover at 25°C for 90 minutes. Thereafter, the cells were transferred to serum-free Schneider's medium and incubated for 3 hours at 25°C. The conditioned medium was centrifuged at 2000 ***g*** for 5 minutes to pellet the cells and then at 100,000 ***g*** for 30 minutes to remove cellular debris. Before use in experiments, the conditioned media were concentrated five times on a Centriprep 50 column (Amicon Inc., MA, USA). The anti-β-catenin antibody was purchased from Zymed Laboratories Inc, CA, USA.

RESULTS

Isolation and characterisation of Wnt-5a transfectants

It has previously been shown that Wnt-5a expression is induced during some phases of mouse mammary gland development and modulation (Weber-Hall et al., 1994). To explore the features associated with Wnt-5a induction**,** we overexpressed this gene in the HB2 mammary epithelial cell line. To differentiate between overexpression and endogenous expression of Wnt-5a protein, we transfected the cells with a HA-tagged Wnt-5a cDNA (Fig. 1A). Using an anti-human Wnt-5a antibody that we developed in rabbits, we estimated that transfection of Wnt-5a resulted in a total threefold elevation of this protein compared to control cells transfected with an empty vector (Fig. 1B). More importantly, we repressed the endogenous expression by transfecting HB2 cells with a mammalian vector that constitutively expresses Wnt-5a antisense RNAs. The isolated antisense populations proved to

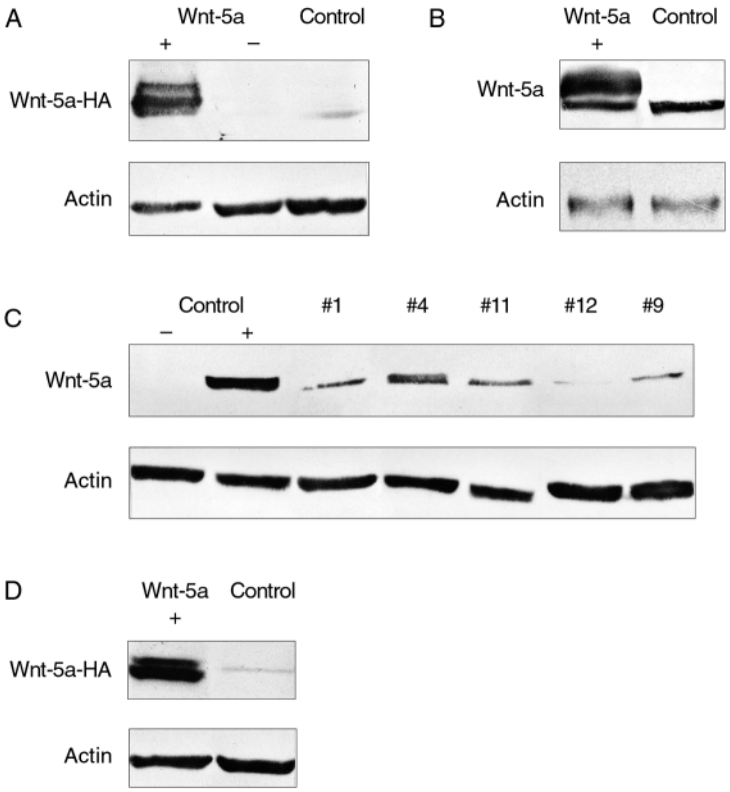

Fig. 1. Expression of Wnt-5a protein in various cell populations. (A) Wnt-5a-HA fusion protein was detected in the lysates of Wnt-5a-HA-overexpressing cells, using an anti-HA antibody (lane 1), but was not found in the lysates of Wnt-5a antisense (lane 2) or control (lane 3) cells. (B) The content of Wnt-5a protein (Wnt-5a-HA) in Wnt-5a-overexpressing cells detected by an anti-Wnt-5a antibody, which recognises both endogenous and overexpressed Wnt-5a. (C) Examples of Wnt-5a repression in antisense transfectants: lane 1 shows the absence of Wnt-5a protein in a lysate of NIH 3T3 cells (negative control); lane 2, endogenous expression of Wnt-5a protein in a lysate of control cells; lanes 3–7, repression of endogenous Wnt-5a protein in five antisense transfectants (detected with the anti-Wnt-5a antibody), as compared to endogenous levels in control lane 2. (D) Expression of Wnt-5a-HA in lysates of the MCF-7 cells transfected with Wnt-5a (lane 1) and control MCF-7 cells (lane 2). All membranes were subsequently stripped and reprobed with an anti-actin antibody to confirm protein integrity and equal loading.

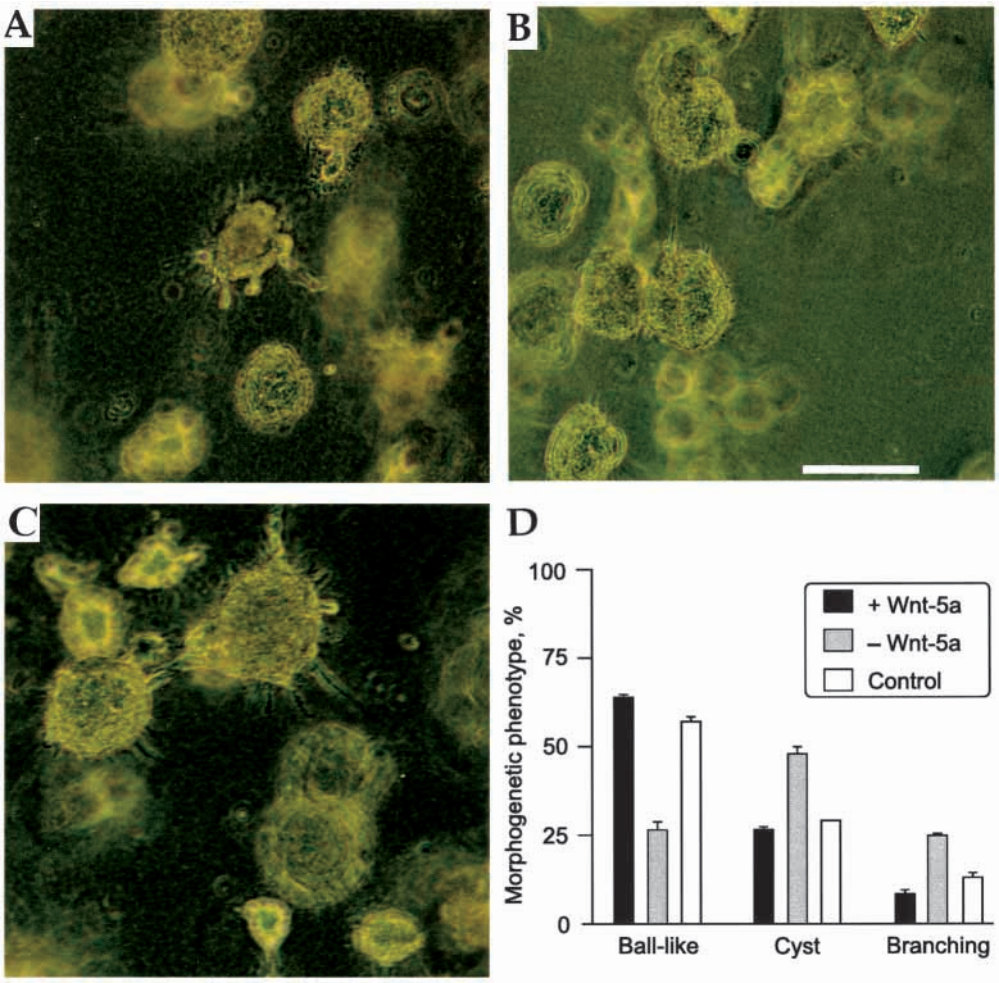

Fig. 2. Formation of morphogenetic structures by various HB2 cell populations. The structures were designated ball-like, cyst shaped, and branching, as described in Materials and Methods. The micrographs show the structures formed by control cells (A), Wnt-5a-overexpressing cells (B) and Wnt-5a antisense cells (C). One hundred morphogenetic structures of each cell population were examined and classified on each of the triplicate plates. Mean values were calculated and plotted on the graph (D). The values represent the mean and standard deviation of determinations and are expressed as percent of the scored structures. Bar, 80 μm.

retain 10-20% of the endogenous Wnt-5a protein level (Fig. 1C). We also used an anti-HA antibody to analyse and ascertain the expression of the Wnt-5a-HA fusion protein in MCF-7 stable transfectants (Fig. 1D).

Although the isolated Wnt-5a-overexpressing cells exhibited a similar growth and morphology as the control cells, repression of Wnt-5a protein changed the appearance of the cells from cuboidal to more semi-elongated (data not shown). This phenotypic alteration was similar to that observed by Olson and Gibo, when they inhibited Wnt-5a expression in the mouse mammary cell line C57MG (Olson and Gibo, 1998).

Repression of Wnt-5a protein enhances intensity of cyst and branch formation

When breast cells are cultured on three-dimensional matrices, they re-acquire many of differentiated properties, including the capacity to assume a polarised morphology and to synthesise and deposit ECM components (Streuli et al., 1991). In the presence of appropriate factors these cultures undergo multicellular restructuring, which resembles mammary morphogenesis in vivo (Streuli and Bissell, 1990).

Previously a collagen type I system has been used to induce morphogenesis of mammary HB2 cells (Berdichevsky et al., 1994). Three major events are characteristic of morphogenesis of HB2 cells in collagen: (1) formation of compact spheres, which we call ball-like; (2) extension of cell processes from the surfaces of these spheres, referred to as cysts and (3) extension of branches, especially in response to treatment with HGF. This cytokine is one of the most important soluble factors involved in epithelial morphogenesis (Gherardi and Stoker, 1991; Niranjan et al., 1995). In several studies, seeding cells in three-dimensional gels has been used as an experimentally traceable system to examine the role of cell-matrix interactions for cell adhesion and migration, as well as for gene expression (Shannon et al., 1987; Streuli et al., 1991; Keely et al., 1995). We used this system to assess a possible connection between the level of Wnt-5a protein and the migratory capacity of HB2 cells measured as the ability to form cysts and branches in collagen gels.

Quantification of the phenotypes produced in the absence of HGF revealed that Wnt-5a-overexpressing and control cells readily produce ball-like phenotypes. By comparison, antisense cells generated twice as many cyst-shaped and branching structures as did Wnt-5a-overexpressing or control cells (Fig. 2).

We also supplemented the culture media with HGF to further stimulate cell migration. We found that, after exposure to HGF, Wnt-5a-overproducing and control cells displayed only small numbers of branches. However, formation of cysts and branches was twice as common in the antisense cells as in the control cells. Interestingly, branch formation was reduced six times more effectively in the Wnt-5a-overexpressing cells than in the antisense cells (Fig. 3).

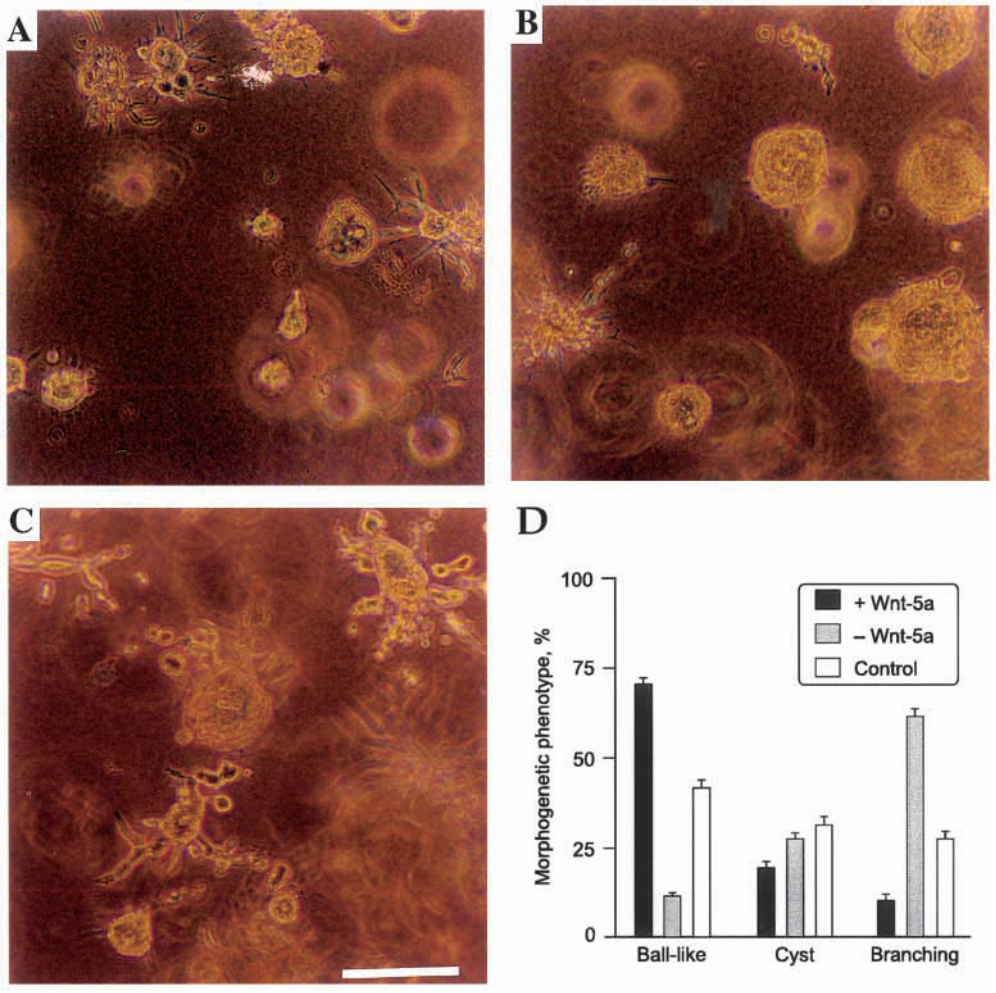

Fig. 3. Formation of morphogenetic structures by various HB2 cell populations in the presence of 10 ng/ml human recombinant HGF. The micrographs show the structures produced by control cells (A), Wnt-5a-overexpressing cells (B) and Wnt-5a antisense cells (C). (D) The morphogenetic structures were quantified as described in Fig. 2D. Values represent the mean and standard deviation of determinations made on triplicate plates and are expressed as percent of the scored structures. Bar, 80 μm.

Repression of Wnt-5a protein impairs cell-to-collagen adhesion

A crucial factor that determines the rate of cell migration is the degree of adhesiveness of cells to their ECM substrata (DiMilla et al., 1991). Thus, the elevated capacity of antisense cells to penetrate collagen gels and form branches could be a consequence of impaired cell-to-collagen attachment. To determine whether this was actually the case, we cultured cells on collagen gels. Initially we observed that, under identical conditions, the Wnt-5a-overexpressing and control cells retained the features of cells cultured on tissue culture plates (Fig. 4A), whereas the antisense cells displayed a scattered phenotype (Fig. 4B). The fact that this scattered phenotype was only observed in cells growing on collagen, but not in cells growing on tissue culture plates, is in agreement with the finding that ECM plays a significant role in regulating the mammary cell phenotype (Streuli and Bissell, 1990; Streuli et al., 1991).

To determine a possible association between Wnt-5a protein levels and cell-to-matrix binding ability, various cell populations were subjected to a collagen-binding assay. After 2 hours of incubation on collagen type I, many antisense cells were detached upon washing, which implies weakened adhesion in response to Wnt-5a repression. In this experiment, overproduction of Wnt-5a protein resulted in a twofold increase in cell-to-collagen adhesion (Fig. 5A). The fact that repression of Wnt-5a impaired, but did not eradicate, the attachment of antisense cells to the collagen suggests that the absence of Wnt-5a protein influences certain collagen receptors, while others remain unaffected.

Wnt-5a repression does not affect the expression or activity of α2β1 and α3β1 integrin receptors

It has previously been shown that α2β1 and α3β1 integrin receptors are present on the surface of HB2 cells and that the

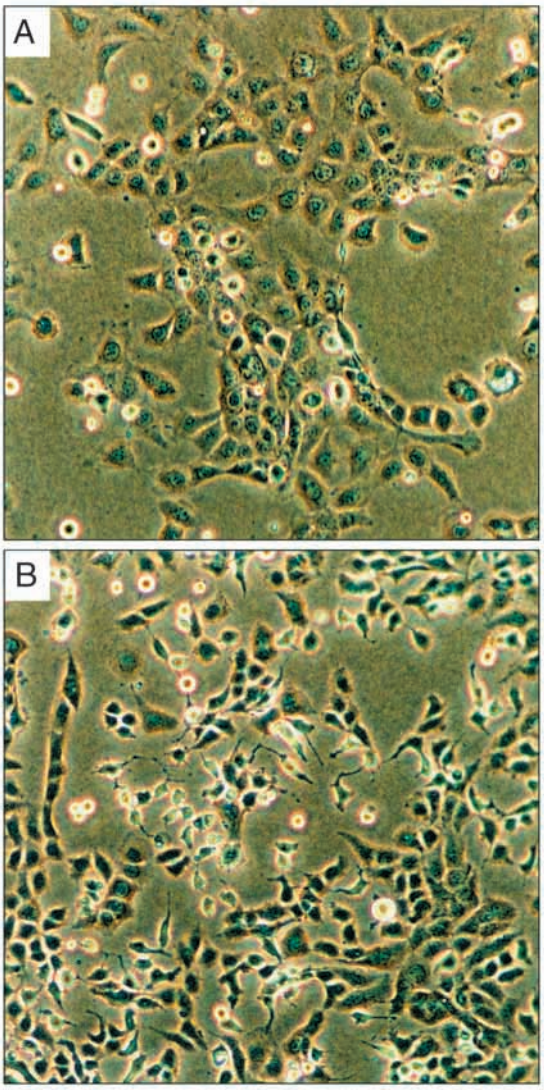

Fig. 4. Morphological characteristics of Wnt-5a antisense cells grown on collagen type I gels. (A) Morphology of control cells grown on collagen matrices. (B) The scattered morphology of antisense cells grown under identical conditions to control cells. The antisense cells shown in panel B were from transfectant 1; transfectants 9 and 12 showed identical morphology.

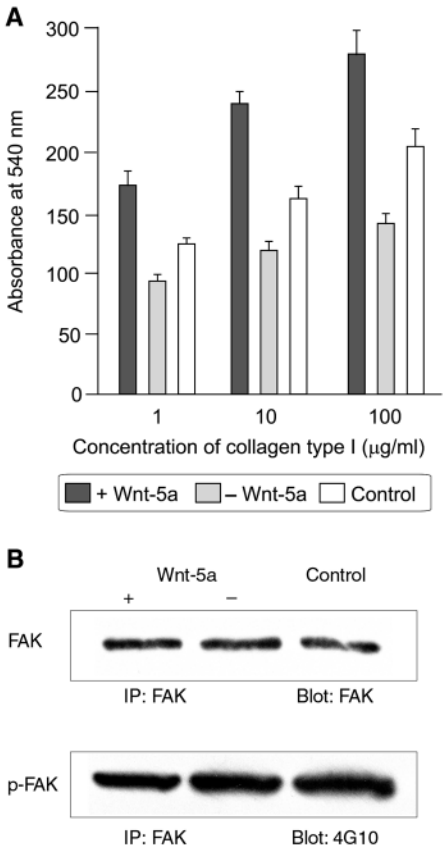

Fig. 5. The cell-to-collagen binding ability of various cell populations. (A) The data represent the average number of cells that adhered to a matrix containing various concentrations of collagen type I during 2 hours incubation. The values are given as the mean and standard deviation of determinations done on triplicate wells assayed in parallel. (B) Tyrosine phosphorylation of FAK in cells with different Wnt-5a protein levels. Upper panel: level of FAK expression in Wnt-5a-overexpressing cells (lane 1), in antisense cells (lane 2), and in control cells (lane 3). Lower panel: level of FAK phosphorylation in various cell populations as indicated in B. The molecular weight of FAK was estimated to 125 kDa by comparing with a molecular standard weight.

branching morphogenesis of these cells can be inhibited by modulating integrin function. For example, activating antibodies to the β1 integrin subunit, which enhances cell-matrix interaction, inhibits the branching of HB2 cells and reduces the motile response of these cells to HGF (Alford et al., 1998). Hence, to determine whether the effects of Wnt-5a protein on cell adhesion and migration are accompanied by modifications in the repertoire of α2β1 and α3β1 integrin receptors, we biotinylated these receptors and, using specific antibodies, quantified them on the surface of the different cell populations. The results showed that all studied cell populations expressed equal amounts of the two receptors on their surfaces (data not shown).

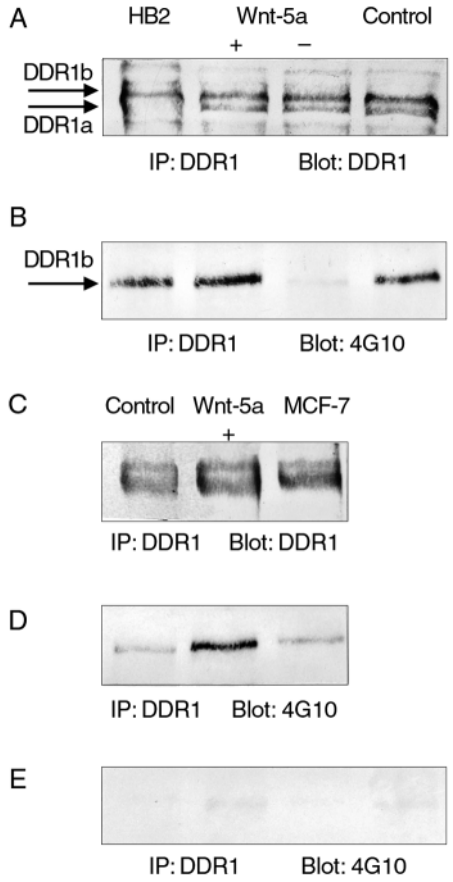

Fig. 6. DDR1 expression by various HB2 cell populations. (A) DDR1 protein level detected in lysates of the following: non-transfected parental HB2 cells (lane 1), Wnt-5a-overexpressing cells (lane 2), Wnt-5a antisense cells (lane 3), and control cells (lane 4). (B) The tyrosine phosphorylation status of DDR1 receptors in various cell populations grown on collagen. Sample loading was done in the same order as in A. (C) DDR1 expression in MCF-7 control (lane 1), Wnt-5a-expressing MCF-7 (lane 2), and untransfected parental MCF-7 (lane 3) cells. (D) The tyrosine phosphorylation status of DDR1 receptors in various MCF-7 cell populations; sample loading was done in the same order as in 6C. (E) Tyrosine phosphorylation status of DDR1 receptors in cells grown on uncoated tissue culture plates; sample loading was done in the same order as in A. The molecular weight of DDR1 was estimated to 125 kDa by comparing with a molecular standard weight. The anti-DDR antibody reacts with isoform b/c (upper band) and with isoform a (lower band) of the DDR1 protein, but only isoform b/c is tyrosine phosphorylated.

Because the expression of integrins does not necessarily reflect the activity of these molecules, we analysed the degree of phosphorylation/activation of focal adhesion kinase. The phosphorylation/activation of this kinase is known to be an initial event in integrin-induced signal transduction and the subsequent regulation of the cytoskeleton, as well as of gene expression (Schlaepfer and Hunter, 1998; Giancotti, 2000). Here, we have used phosphorylation of FAK as an indicator of α2β1 and α3β1 integrin signalling. As shown in Fig. 5B, we found that FAK was phosphorylated on tyrosine residues to a similar extent in all cell populations, implying that the impaired interactions between Wnt-5a antisense cells and collagen are not due to a disruption of α2β1 and α3β1 integrin activity.

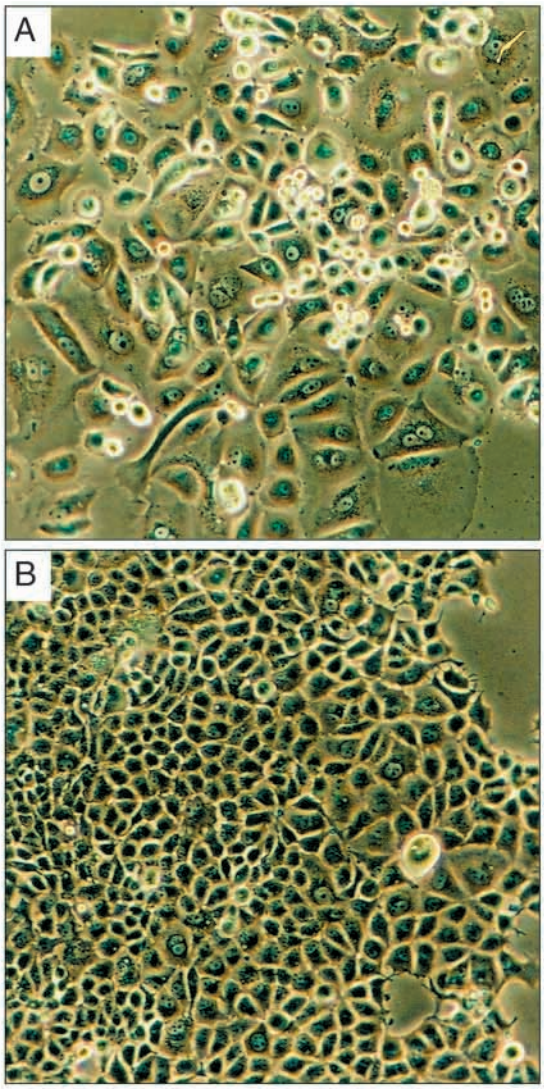

Fig. 7. Expression of Wnt-5a improves the morphology of MCF-7 breast cancer cells to a more normal phenotype. (A) Morphology of control MCF-7 cells transfected with the vector alone. (B) Morphology of MCF-7 cells expressing Wnt-5a protein. The Wnt-5a-expressing MCF-7 cells grow as islands containing tightly associated cells and therefore they appear to have a higher density than that of control cells, despite the fact that an equal number of both cell populations were plated and grown for 16 hours.

Wnt-5a protein is required for collagen-induced DDR1 phosphorylation

Several types of collagen serve as ligands for non-integrin DDR receptors and induce dimerisation and subsequent transphosphorylation of DDR1 and DDR2. Expression of the DDR1 receptor is essentially restricted to epithelial cells, whereas DDR2 is expressed in surrounding stromal cells (Alves et al., 1995). In our experiments, the DDR1 protein was endogenously expressed in parental HB2 cells. Furthermore, we found that the DDR1 protein was produced at a similar level in Wnt-5a-overexpressing, control and antisense cells grown on collagen (Fig. 6A). However, repression of the Wnt-5a protein led to significantly lower tyrosine phosphorylation of DDR1 receptors in the Wnt-5a antisense cells (Fig. 6B). The Wnt-5a-overexpressing cells showed a DDR1 phosphorylation level comparable to that detected in the parental HB2 cells, which suggests that the endogenous expression of Wnt-5a is sufficient to enable collagen-induced phosphorylation of DDR1 receptors.

To confirm that the Wnt-5a protein is necessary for phosphorylation of DDR1 receptors, we transfected a human breast cancer cell line, MCF-7, which has lost the ability to express Wnt-5a mRNA (Lejeune et al., 1995). We found similar levels of DDR1 protein in control, Wnt-5a-expressing and parental cells (Fig. 6C). However, only the Wnt-5a-expressing MCF-7 cells showed a significant tyrosine phosphorylation of DDR1 receptors (Fig. 6D). Interestingly, the expression of Wnt-5a protein in MCF-7 cells not only enabled phosphorylation of DDR1 receptors in the presence of collagen, but it also improved several phenotypic aspects of these cancer cells, such as morphology, tight association between the cells, and contact inhibition at cell confluence (Fig. 7).

DDR1 appears to exist in three isoforms, designated DDR1a, DDR1b and DDR1c (Vogel, 1999), hence we performed experiments to determine which isoform is phosphorylated in the presence of the Wnt-5a protein. We separated DDR1a from DDR1b by using a long-run gel. However, we were unable to separate DDR1b from DDR1c because these two proteins have almost the same molecular weight. Nonetheless, our results do indicate that the effects of the Wnt-5a protein on DDR1 phosphorylation are limited to either DDR1b or DDR1c, or to both of these isoforms.

DDR1 phosphorylation requires the presence of both Wnt-5a and collagen

Collagen type I matrix has been identified as a ligand for the DDR1 receptors (Vogel et al., 1997). To ascertain whether Wnt-5a-mediated effects on DDR1 receptor phosphorylation would persist in the absence of collagen, we examined DDR1 phosphorylation in cells grown on uncoated tissue culture plates for 16 hours. Cells adhering to a noncollagen coated surface exhibit a phosphorylation level of DDR1 that is almost undetectable (Fig. 6E). Thus, it appears that both collagen and Wnt-5a protein are necessary for DDR1 phosphorylation, that is, both repression of Wnt-5a in antisense cells and elimination of collagen from the matrix of Wnt-5a-expressing cells impede DDR1 phosphorylation. This suggests that Wnt-5a protein serves as a co-factor, necessary for the collagen-induced phosphorylation/activation of DDR1 receptors.

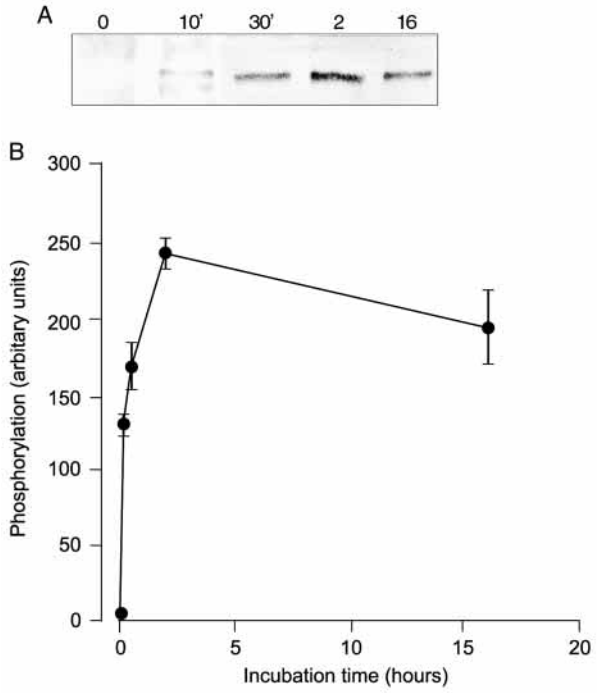

Fig. 8. Kinetic analysis of phosphorylation of DDR1 receptors. (A) Control cells grown on collagen for various periods of time. The cells were lysed and immunoprecipitated with an anti-DDR1 antibody, and the tyrosine phosphorylation status of the DDR1 receptors was determined by immunoblotting with an anti-tyrosine antibody (4G10). (B) DDR1 phosphorylation during the indicated periods of time. The values represent the mean and standard deviation of determinations done on triplicate wells assayed in parallel.

Kinetic analysis of DDR1 activation in the presence of Wnt-5a protein

To determine how rapidly the DDR1 receptor is phosphorylated in the presence of Wnt-5a protein, we performed a kinetic analysis, which entailed growing the cells on collagen for different periods of time. Because the Wnt-5a-overexpressing and control cells displayed equal levels of DDR1 phosphorylation (Fig. 6B), only control and antisense cells were subjected to kinetic analysis. Tyrosine phosphorylation of DDR1 receptors occurred after only 30 minutes of incubation on collagen and was sustained after 16 hours (Fig. 8A,B). The slow activation of DDR1 kinase, as compared to conventional tyrosine kinases (which are activated very rapidly) may reflect a gradual and slow interaction of DDR1 receptors with the collagen surface, or a gradual deposition of Wnt-5a necessary for mediation of a full signalling response. Concerning the Wnt-5a antisense cells, they did not exhibit DDR1 phosphorylation regardless of the incubation time (data not shown).

The Wnt-5a-dependent phosphorylation of DDR1 receptors is insensitive to cytosolic accumulation of β-catenin

As mentioned earlier, Wnt-5a can stimulate the Wnt/β-catenin signalling pathway (Miller et al., 1999). Here, we used a *Drosophila* cell line that expresses a Wg cDNA and conditions culture medium with active soluble Wg protein (Fig. 9A; van

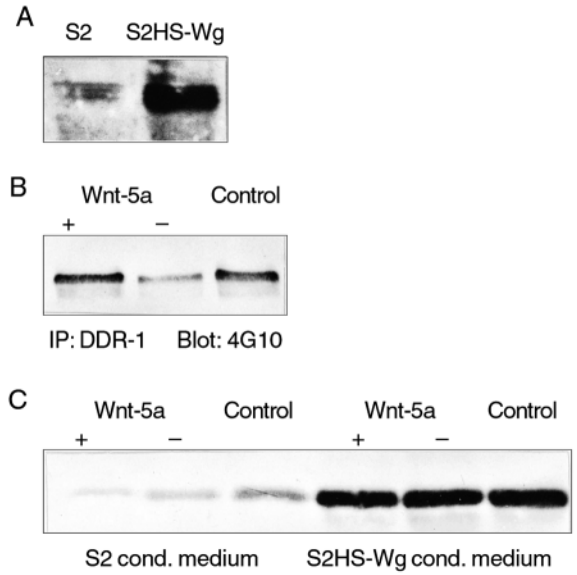

Fig. 9. Effects of β-catenin accumulation on DDR1 phosphorylation. (A) The presence of secreted Wg protein in medium conditioned by S2HS-Wg compared to that conditioned by S2 control cells. (B) Extracts from various cell populations, grown on collagen for 30 minutes in the presence of Wg protein, were prepared and analysed for phosphorylation of DDR1 receptors. Double extracts were also prepared in parallel to detect induction of β-catenin protein. (C) Accumulation of β-catenin in response to incubation with concentrated Wg-conditioned medium, ensured by exposing cells to medium conditioned for 30 minutes by control S2 cells (lanes 1-3) or Wg-producing cells (lanes 4-6).

Leeuwen et al., 1994) to explore the connection between the Wnt-5a-mediated effect on DDR1 phosphorylation and accumulation/activation of cytoplasmic β-catenin protein. Other investigators (Ramakrishna and Brown, 1993; Cook et al., 1996) have found that Wg-conditioned medium can activate the Wnt-1 (mammalian homologue of Wg) signalling pathway in mammalian cells.

The cells were grown on collagen and incubated with Wg-conditioned medium for 30 minutes, a period adequate for detection of DDR1 phosphorylation. We did not prolong the treatment because the activity of the Wg protein declines after 30 minutes of incubation with cells (Cook et al., 1996). We found that the DDR1 receptors remained unphosphorylated in the antisense cells, whereas Wnt-5a-producing cells exhibited a DDR1 phosphorylation level similar to that observed in non-β-catenin-induced cells (Fig. 9B). As shown in Fig. 9C, exposure of the various cell populations to Wg soluble protein led to the accumulation of cytoplasmic β-catenin. Inasmuch as a similar level of β-catenin was present in the cytoplasm of all cell populations, and the β-catenin protein was induced to about the same extent in all studied cells, we conclude that the failure to phosphorylate DDR1 in Wnt-5a antisense was not related to the Wnt/β-catenin signalling pathway.

DISCUSSION

In the present study, we have shown that repression of the Wnt-5a protein enhanced the capacity of antisense cells to branch and penetrate through collagen matrices spontaneously, i.e. in the absence of exogenous stimuli. The exposure of the cells to the HGF, which stimulates cell migration, promoted branching in all cell populations, although this effect was most prominent in the antisense cells. The endogenous level of Wnt-5a protein in control cells reduced cell migration compared to antisense cells, but a threefold overproduction of this protein, in excess of the endogenous level, proved to be necessary to obtain an almost full inhibitory effect on HGF-mediated cell migration. Thus, induction of Wnt-5a protein might be an important mechanism for counteracting the HGF-induced migration during certain stages of mammary gland development and modulation**.** In support of our data regarding an anti-migratory effect of Wnt-5a, it has been shown that ectopic expression of XWnt-5a in *Xenopus* embryos could block the activin-stimulated morphogenetic movements of the blastula cap (Moon et al., 1993).

The enhanced motility of Wnt-5a antisense cells inside collagen gels could be a direct consequence of altered cell-to-collagen adhesion. We tested that possibility and found that an impaired cell-to-collagen interaction could indeed explain why antisense cells exhibit elevated cell migration in collagen. This encouraged us to search for putative collagen receptors that might have been affected in response to repression of the Wnt-5a protein. We observed no quantitative differences between various cell populations in regard to the expression of α2β1 and α3β1 integrin receptors or to their activity, measured as the extent of FAK phosphorylation. However, we did discover a strong association between the phosphorylation/activity of DDR1 receptors and the level of Wnt-5a protein. More precisely, under identical experimental conditions, DDR1 receptors were phosphorylated in both Wnt-5a-overexpressing and control cells, but not in the Wnt-5a antisense cells, despite the presence of similar levels of DDR1 protein. In support of our finding that repression of Wnt-5a hinders DDR1 receptor phosphorylation but does not affect the status of integrin receptors, it has recently been demonstrated that signals from DDR1 and β1 integrin receptors are transmitted through distinct intracellular pathways (Vogel et al., 2000).

To further ascertain that the presence of Wnt-5a protein is essential for DDR1 phosphorylation, we transfected non-Wnt-5a-expressing MCF-7 breast cancer cells with a mammalian vector carrying Wnt-5a cDNA. Interestingly, production of Wnt-5a protein in MCF-7 cells was accompanied by an increased ability to phosphorylate DDR1 receptors and by improvement of the cancer cell phenotype. It is surprising that phosphorylation of DDR1 receptors was associated with a less malignant phenotype of MCF-7 cells, because it has been reported that the message level of the DDR1 receptor is upregulated in breast cancer (Barker et al., 1995). Nonetheless, we did not find any correlation between the level of protein expression and the degree of phosphorylation of DDR1 receptors in any of the cell types we investigated.

We have noticed that both collagen matrices and Wnt-5a protein are necessary for DDR1 activity. It is possible that collagen is required for sequestration, presentation or reception of the secreted Wnt-5a signals, which in turn affect DDR1 phosphorylation. In support of this, it has been shown that, in addition to Fz receptors, other components of the outer cell membrane or non-integrin ECM receptors (e.g. proteoglycans) are needed for Wnt signalling. For instance, the removal of

heparin sulfate has been found to block XWnt-8 activity in *Xenopus* embryos, indicating the importance of a Wnt-ECM interaction (Itoh and Sokol, 1994).

Our results show that phosphorylation of DDR1 receptors did not occur in the presence of Wnt-5a alone, but required the presence of collagen too. Because phosphorylation of DDR1 receptors is gradually induced in the presence of Wnt-5a, it is possible that secreted Wnt-5a acts as a co-factor necessary for collagen-induced activation of DDR1 receptors. In support of this idea, it was recently demonstrated that, in addition to collagen, another – as yet unidentified – component is required for initiation of full signalling of the DDR1 kinase domain in PC12 cells (Foehr et al., 2000). However, the fact that the collagen ligand is required for the induction of DDR1 phosphorylation argues against a direct receptor cross-talk between Fz-5 and DDR1 receptors similar to that identified between integrins and RTKs (Clark and Brugge, 1995).

Despite many reports on integrins as matrix binding receptors, little is known about a similar role for DDR receptors. In the present study we have shown that Wnt-5a repression impairs both cell-to-collagen binding and collagen-induced DDR1 phosphorylation. It is possible that DDR1 receptors do not function as mechanical adhesion receptors but that they instead sense the contact between cells and collagen matrices, which then initiates intracellular signalling and cytoskeletal rearrangements. Although this issue requires further investigation, our data suggest a relationship between Wnt-5a-dependent DDR1 phosphorylation and adhesion of breast epithelial cell onto collagen.

Accumulation of the β-catenin protein in the cytoplasm is one of the most important events in Wnt signalling (Miller and Moon, 1996). The accumulated β-catenin enters into the cell nucleus and increases the expression of certain target genes (Behrens et al., 1996). In the present study, we investigated whether the Wnt-5a-dependent phosphorylation of DDR1 was mediated through accumulation/activation of cytosolic β-catenin by activating the canonical Wnt signalling pathway with a Wg soluble protein. We found that addition of Wg soluble protein did not restore the ability of antisense cells to phosphorylate DDR1 receptors. Furthermore, the level of cytosolic β-catenin remained unaffected in all cell populations regardless of the expression level of Wnt-5a protein. These data indicate that activation of the Wnt/β-catenin pathway has little or no impact on DDR1 phosphorylation. After excluding a role for canonical Wnt signalling in phosphorylation/activation of DDR1 receptors, it is feasible to assume that the effect of Wnt-5a on DDR1 phosphorylation is mediated through the non-canonical Wnt/Ca^{2+} signalling pathway. This signalling pathway is initiated by the binding of Wnt ligands to specific Frz receptors and may function through heterotrimeric G-proteins (Sheldahl et al., 1999). Activation of this pathway leads to release of intracellular Ca^{2+} and stimulates activation of protein kinase C and Ca^{2+}/calmodulin-dependent protein kinase II (Kühl et al., 2000). Although identification of the Wnt/Ca^{2+} signalling as a β-catenin-independent pathway has served as a paradigm for elucidating the role of many Wnt genes in development, it remains unclear whether the cellular and embryonic responses are specific to activation of this signalling pathway. Further elucidation of Wnt/Ca^{2+} signalling will not only clarify this issue but also provide guidelines for investigating whether the activation of this signalling pathway mediates the effect of Wnt-5a on DDR1 phosphorylation/activation.

Taken together, the present study reports the following new features. First, it suggests that the DDR1 receptor is a new molecular target for noncanonical Wnt signalling. Second, it documents that Wnt signalling depends on the extracellular matrix. Third, it provides an explanation to support studies in developmental systems by showing a role for Wnt-5a in the regulation of cell adhesion and migration. These properties may be relevant for mediation of Wnt-5a effects during normal mammary gland modulation, whereas loss of these properties can contribute to the development of a malignant phenotype with enhanced capacity to invade.

This work was supported by the Swedish Cancer Foundation, the Swedish Society for Medical Research, the John and Augusta Persson Fund for Medical Research, the Research Foundations of Malmö and Lund University Hospitals and the Royal Physiographic Society in Lund.

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