Wnt-5a mRNA translation is suppressed by the Elav-like protein HuR in human breast epithelial cells

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ABSTRACT

Wnt-5a is a non-transforming Wnt protein. Since Wnt-5a mRNA and protein levels differ within and between tumours, the potential of Wnt-5a as a prognostic factor has been debated. We have previously shown that the lack of Wnt-5a protein is a predictor of shorter disease-free survival in human breast cancer. Recently, however, we also showed that the breast tumours lacking Wnt-5a protein had a high or normal level of Wnt-5a mRNA that might explain the discrepancies in previous studies. We here report that Wnt-5a is regulated at the post-transcriptional level. The regulation was mediated by the Embryonic Lethal Abnormal Vision (ELAV)-like protein HuR, which inhibited translation of Wnt-5a when bound to highly conserved AU-rich sequences in the 3'-untranslated region of the Wnt-5a mRNA molecule, as shown by both HA-tagged Wnt-5a- and Luciferase-Wnt-5a-3'-UTR reporter assays. The HuR-dependent inhibition of Wnt-5a was supported by the fact that active HuR is located in the cytoplasm in invasive human breast tumours and that hypoxia-induced activation of HuR inhibits translation of both Luciferase-Wnt-5a-3'-UTR and endogenous Wnt-5a protein. We propose that the lack of Wnt-5a protein expression in invasive human breast tumours is caused by a HuR-mediated suppression of Wnt-5a mRNA translation.

INTRODUCTION

Wnt proteins influence multiple developmental processes and have also been implicated in carcinogenesis, including the development of breast cancer (1–4). Depending on which signalling pathway the Wnt proteins activate, they are referred to as canonical (Wnt/β-catenin) or non-canonical Wnt proteins [reviewed in (5)]. Wnt-5a has been characterized as a non-canonical (5–10) and non-transforming Wnt protein (11) that is important for cellular adhesion, migration, planar cell polarity and convergent extension movements.

We have previously shown that expression of Wnt-5a is a predictor of longer disease-free survival in human breast cancer (12,13). Expression of Wnt-5a in mammary cells confers a more differentiated phenotype (14), an increased adhesion and a decreased motility (15,16). Recent evidence links both canonical (Wnt/β-catenin) and non-canonical (Wnt/Ca2+) pathways to tumour invasion and metastasis (17). However, the importance for Wnt-5a signalling in metastatic processes is debated and conflicting results concerning the expression levels of Wnt-5a in different invasive tumours can be found in the literature (9,12–18). There are probably two reasons for this; first, an abnormal protein expression level of Wnt-5a can affect adhesion and metastasis both when being over-expressed (loosely attached cells; malignant melanoma) and repressed (firmly attached cells; breast cancer). Thus, loosely attached cells will display an increased capacity to migrate upon increased adhesion (Wnt-5a signalling) while firmly attached cells will increase their migration upon a decreased adhesion (lack of Wnt-5a signalling). Second, most early studies concerning the role of Wnt-5a in cancers have analysed the expression levels of Wnt-5a mRNA and not protein. In a recent study, we show that breast tumours lacking Wnt-5a protein expression have a high or normal level of Wnt-5a mRNA as judged by in situ hybridization (13). This means that studies concerning Wnt-5a expression can only be judged by protein analysis of Wnt-5a. It also indicates that Wnt-5a expression probably is regulated at the post-transcriptional level.

Post-transcriptional regulation can be achieved by different means and includes mRNA processing, nucleo-cytoplasmic export, mRNA localization, mRNA stabilization and translational regulation [reviewed in (25,26)]. These processes are influenced by the conserved sequence elements located in the untranslated regions (UTRs) of the mRNA molecules.

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and the specific mRNA binding proteins that can bind to and affect its properties [reviewed in (26,27)]. Proteins bind to UTRs via specific RNA-recognition motifs (RRM) and the mRNA binding sites are usually located in the 3’ ends of mRNAs. The sites are most often AU-rich or U-rich sequences and the binding proteins are therefore referred to as AU-rich element binding proteins (ARE-binding proteins). ARE-binding proteins can stabilize (AUBF, Elav-like family) or destabilize (AUF1, TTP and KSRP) mRNAs (25–28). One common cellular mechanism is thus to produce unstable mRNAs that can be stabilized upon binding of specific RNA-binding proteins (27). This way, the output of mRNAs for a specific gene can be constant, but will yield high levels of protein only when the mRNAs are stabilized and subsequently translated. Another less well-described mechanism is to suppress translation (TIAR, TIA-1 and CUGBP2) (25,29,30).

The Elav-like family of ARE-binding proteins include the proteins HuR, HuD, HuC and Hel-N1 (27,31). They are most well known as mRNA stabilizing proteins that bind AREs in the 3’-untranslated regions (3’-UTRs) of unstable mRNAs. They act as nuclear-cytoplasmic shuttle proteins that predominantly are expressed in the nucleus but shuttle out into the cytoplasm upon initiation of cell signalling [e.g. by p38 MAPK, Wnt/b-catenin and Notch signalling; reviewed in (26)] or under stress conditions, such as hypoxia (26,32). Recent studies reported that cytoplasmic localization of HuR is associated with a high tumour grade or invasiveness in ovarian and breast cancers (33–36). These data are particularly interesting in relation to the observed shorter disease-free survival in breast cancer patients with reduced Wnt-5a protein expression (12,13). In this study we investigated why the protein- but not mRNA levels of the prognostic marker Wnt-5a, are decreased in invasive ductal breast cancer.

**MATERIALS AND METHODS**

**Constructs**

The cDNA3 vector was obtained from Invitrogen and the pGL3-Luciferase and cytomegalovirus (CMV)–Renilla vectors from Promega. The 3’-UTR parts corresponding to Wnt-5a 3’-UTR 1462–1528 (1–66 bp), 1462–1645 (1–183 bp) and 1462–1720 (1–258 bp) were PCR-amplified from cDNA kindly provided by Prof. A. Harris, using primers with 5’ XbaI sites in the sense primer (5’-ctcattgattgtcctgaccaccagctacgcc-3’) and antisense primers (5’-ggttattatataggagttgctgtgacacctgcttactggcttatcg-3’) and antisense primers (5’-ggttattatataggagttgctgtgacacctgcttactggcttatcg-3’) and antisense primers (5’-ggttattatataggagttgctgtgacacctgcttactggcttatcg-3’). The purified fragments were digested with XbaI and the pcDNA3 vector with XbaI/Apal (Apal site blunt-ended).

The constructs were sequenced and subsequently digested with EcoRI/EcoRV (Wnt-5a), HindIII/XbaI (constructs designated L1 and L3) and KpnI/XbaI (constructs designated L and L2) and ligated to Wnt-5a (EcoRI/EcoRV; pcDNA3- HA-Wnt-5a) as a kind gift from Dr M. Sen, or Luciferase (HindIII/XbaI or KpnI/XbaI; pGL3).

**Cells and culture conditions**

The HB2, non-cancerous mammary epithelial cell line, is a subclone of the MTSV-7 cell line originating from the laboratory of Dr J. Taylor-Papadimitriou (ICRF, UK) (27). The human breast cancer cell lines MCF-7 and T47D were purchased from ATCC. The HB2 cells were cultured in DMEM supplemented with 10% fetal bovine serum, 10 μg/ml bovine insulin and 5 μg/ml hydrocortisone. The other cell lines were cultured in DMEM supplemented with 10% fetal bovine serum. All cell cultures were kept at 37°C in a humidified atmosphere with 5% carbon dioxide.

**Western blotting**

Adherent cells grown to subconfluence on tissue culture plates were washed with phosphate-buffered saline (PBS) and lysed in 250 μl of 5x concentrated Laemmli buffer supplemented with 100 mM DTT. The samples were boiled for 30 min and then subjected to electrophoresis on 10% SDS–polyacrylamide separating gel. The separated proteins were electrotransferred to PVDF membranes, which were incubated with 5% non-fat milk or PBS supplemented with 0.05% Tween (PBS-T) for 1 h at room temperature. Thereafter, the membranes were probed with antibodies against Wnt-5a produced in our lab as described previously (12) (1:2000), anti-HuR [Santa Cruz (1:1000)] or anti-HA [ Molecular probes (1:5000)], respectively, for 1 h at room temperature. The separated protein bands were visualized by incubating the membranes with horseradish peroxidase (HRP)-conjugated secondary antibodies and ECL. The blots were re-probed with an antibody against β-actin to ascertain equal protein loading.

**RNA electrophoretic mobility shift assay (REMSA) and ultraviolet (UV)-crosslinking**

32P-labelled cRNAs corresponding to the coding region HA-Wnt-5a (ctrl), 1–66 3’-UTR (#1), 1–183 3’-UTR (#2) or 1–258 3’-UTR (#3) were produced using purified PCR-amplified cDNA spanning the T7 site in the pcDNA3 vector (5’-cccagctacgcttacgcc-3’) and antisense primers for each insert (coding region; c.r. 5’-ggaccagttgttgctgatag-3’) and T7 polymerase (Promega) according to the manufacturer’s procedure. The mutated probe was produced in the same way but using primers corresponding to the sense primer spanning the T7 site in the pcDNA3 vector (5’-cccagctacgcttacgcc-3’) and an antisense primer (5’-aatcttagtatagttctgcccaccagctacgcc-3’) and Digoxigenin labelled rUTPs (Roche). For UV-crosslink immunoprecipitation experiments the probes were also labelled with Digoxigenin (Roche). All regents for cDNA production were purchased from Promega, except for 32P-UTP (Amersham). Probes were DNAse treated (Invitrogen), phenol extracted and dissolved in Munro’s buffer ([10 mM HEPES (pH 7.6), 3 mM MgCl2, 40–100 mM KCl, 5% Glycerol, 1 mM DTT]). The cold probe was prepared using rUTP (Promega) instead of radiolabelled-UTP. Nuclear and cytoplasmic extracts were prepared as described previously (37), and diluted in Munro’s buffer (1:5). Each probe (50,000 c.p.m.) was incubated with 18 μl of the extracts for 30 min at room temperature. Subsequently, RNAse T1 (1 U; Promega) and Heparin (5 μg/ml) was added for 5 min each, the samples were diluted in RNA native loading buffer [30 mM Tris–HCl (pH 7.5), 40% Sucrose and 0.2% BFB] and subjected to native PAGE gel (4%) electrophoresis.
The gels were dried and subjected to autoradiography with intensifying screens at −70°C. For cold competition, the cold probe was added in 10-fold excess for 10 min prior to addition of the radiolabelled probe. In the supershift experiments, the antibody was added after 15 min of incubation. UV-crosslinking was performed as described above and further subjected to UV-crosslinking in a Stratalinker (250 mJ/m²) and subsequently analysed using SDS–PAGE (10%) analysis and normal Laemmli-DTT loading buffer. Immunoprecipitation of UV-crosslinked products were performed using normal lysis buffer [100 mM Tris–HCl (pH 7.5), 1% NP-40, 5 mM EDTA, 5 mM EGTA, 50 mM NaCl, 2 mM Na₃VO₄, 20 μg/ml aprotinin, 1 μg/ml leupeptin, 2.5 mM benzamidine and 2 mM Pefabloc® (Roche)] and a control IgG antibody or anti-HuR antibodies (Santa Cruz) together with RNase-inhibitor treated Protein A-Sepharose beads (Amersham) for 1 h. The beads were washed five times in ice-cold wash buffer [50 mM HEPES (pH 7.4), 1% Triton X-100, 0.1% SDS, 150 mM NaCl and 2 mM Na₃VO₄] and were boiled immediately in Laemmli buffer with DTT. For experiments using Digoxigenin labelled probes a DIG luminescent detection kit for nucleic acids (Roche) was used.

**Transient transfections and luciferase assays**

HB2 cells were transfected with Oligofectamine reagent (Invitrogen) according to the manufacturer’s instructions. Transfections were performed using 1 × 10⁶ cells, 2 μg of HA-Wnt-5a-3'UTR constructs and 0.1 μg of CMV–controlled Renilla reporter gene per transfection, or 0.2 μg of Luciferase-3'UTR reporter gene construct and 0.1 μg of CMV–controlled Renilla reporter gene for HuR transfections. Preparation of protein extracts and luciferase assays were performed with a Dual-Luciferase Reporter Assay System (Promega). The luciferase activity was measured using 1:10 of the reactions. For in vitro transcription, [32P]UTP was added to the reactions programmed with 0.1 μg of the Luciferase-3'UTR constructs only and after 15 min the mRNA was precipitated with 10% ice-cold TCA, incubated on ice for 10 min, washed once in ice-cold 10% TCA and three times in ice-cold 95% ethanol. The amount (c.p.m.) of incorporated UTP was measured using a β-counter. Unprogrammed reticulocyte lysate containing the equal amount of [32P]-UTP was used as a control. The results were corrected for the percentage of UTPs in each construct. For in vitro translation experiments using the Transcend™ non-radioactive translation detection system (Promega), 0.5 μg of the Luciferase-3'UTR constructs (L, L1, L2, L3) were used in 20 μl reactions and of these 1 μl were used for SDS–PAGE analysis. For in vitro translation experiments, using HuR pre-programmed rabbit reticulocyte lysates 1 μg of HuR cDNA was added and translated in a 50 μl reaction. Of this reaction 1 μl was added to the 20 μl Luciferase-3'UTR reactions.

**Immunofluorescence**

The cells were seeded on glass coverslips and grown overnight to allow them to adhere. Thereafter, the cells were washed with PBS, fixed in methanol for 10 min and blocked in 3% BSA–PBS for 1 h at RT. Cells were stained with anti-HuR from Santa Cruz (1:500) or isotype control antibodies in 1% BSA–PBS for 1 h at RT, after which the cells were washed five times with PBS and then incubated with secondary anti-rabbit Alexa Flour 488 (1:500) antibodies. Thereafter, the coverslips were washed five times in PBS, mounted in fluorescent mounting medium (DAKO), and examined and photographed in a Nikon Eclipse 800 microscope, using a 60× objective. Images were recorded with a scientific-grade, charge-coupled device (CCD) camera (Hamamatsu) and were analysed with HazeBuster deconvolution software (VayTek).

**RESULTS**

The 3'UTR of Wnt-5a is evolutionarily conserved and contains AU-rich stretches

The human Wnt-5a 3'UTR, as shown in Figure 1A is ~2.5-fold longer than the coding region and more than five times the length of the 5'UTR (38,39). To find possible important RNA–protein-binding sites (AREs) we searched for evolutionarily conserved sequence elements in the 5'UTR and the 3'UTR of the Wnt-5a mRNA. The sequence corresponding to 143–318 bp in the 5'UTR was conserved between dog, mouse, rat and human, but no AREs were found (data not shown). However, the 3'UTR contained probes generated from the HA-Wnt-5a or Luc constructs or with a 32P-labelled oligo specific for Renilla.

**In vitro translation and transcription**

In vitro translation was performed using the programmed T7/SP6 rabbit reticulocyte lysate system (Promega) and addition of 0.1 μg of the Luciferase-3'-UTR construct and Renilla plasmid together with 1 μg cDNA3, HuR or M1 HuR, all according to the manufacturers procedures. The luciferase activity was measured using 1:10 of the reactions. For in vitro transcription rate analysis, [32P]UTP was added to the reactions programmed with 0.1 μg of the Luciferase-3'UTR constructs only and after 15 min the mRNA was precipitated with 10% ice-cold TCA, incubated on ice for 10 min, washed once in ice-cold 10% TCA and three times in ice-cold 95% ethanol. The amount (c.p.m.) of incorporated UTP was measured using a β-counter. Unprogrammed reticulocyte lysate containing the equal amount of [32P]-UTP was used as a control. The results were corrected for the percentage of UTPs in each construct. For in vitro translation experiments using the Transcend™ non-radioactive translation detection system (Promega), 0.5 μg of the Luciferase-3'UTR constructs (L, L1, L2, L3) were used in 20 μl reactions and of these 1 μl were used for SDS–PAGE analysis. For in vitro translation experiments, using HuR pre-programmed rabbit reticulocyte lysates 1 μg of HuR cDNA was added and translated in a 50 μl reaction. Of this reaction 1 μl was added to the 20 μl Luciferase-3'UTR reactions.
sequences that were conserved between chinchilla, mouse, rat and human (shaded in Figure 1B) and many of these harboured putative AREs of class III or class I according to the definition of AREs [reviewed in (25,40); Figure 1C]. In Figure 1B and C the 3'--UTR is renumbered (1–2570 bp) for simplicity. The most conserved region is located 133–192 bp 3' of the STOP-codon. We further analysed the predicted 2D mRNA folding structure (41,42) (http://www.bioinfo.rpi.edu/~zukerm) and the most conserved regions of AU-rich stretches formed hair-pin and loop structures that were preserved (data not shown) indicating that these sequences are AREs with a high probability of binding RNA-binding proteins (43).

Wnt-5a translation is governed by the 3'-UTR

The Wnt-5a mRNA has previously been reported to occur as at least two transcripts (7 and 4 kb) via utilization of two polyadenylation signals located at ~4.4 and 6.8 kb (38,39). To our knowledge however, only the first 4 kb have been sequenced and the role for the 7 kb transcript is at present unknown. Wnt-5a is expressed at low levels in normal human breast epithelial cells (24). As shown in Figure 1D, the non-cancerous human breast epithelial cell line, HB2, primarily produces the 4 kb transcript. To analyse whether the conserved structures in the 3'-UTR were capable of regulating Wnt-5a protein expression at the post-transcriptional
level, we chose the most conserved domains of the Wnt-5a 3′-UTR. The cloned 3′-UTR stretches were either placed down-stream of the HA-tagged Wnt-5a cDNA or the reporter gene Luciferase. Both the Wnt-5a (W in Figure 2A) and the Luciferase (L) gene were driven by the pCMV promoter and contained the bovine growth hormone polyadenylation signal [BGH poly(A)] in order to obtain a basic and unregulated level of translation (Figure 2A). The different constructs were, as described in Figure 2A, controls devoid of the 3′-UTR (W and L), 1–66 bp of the 3′-UTR (W1 and L1), 1–183 bp with the most conserved ARE (W2 and L2) and finally 1–258 bp (W3 and L3).

To analyse whether the AREs regulated Wnt-5a protein expression, we transfected HB2 cells with the different HA-Wnt-5a containing vectors and analysed the protein expression levels by western blot using an anti-HA antibody. As shown in Figure 2B the protein levels of transfected Wnt-5a decreased with increasing length of the 3′-UTR. The effect on protein expression was gene independent since the same results were obtained using the Luciferase expression plasmid series, cotransfected with a CMV-controlled Renilla expression plasmid (Figure 2C). The experiments were performed as several separate experiments (n = 12), using different preparations of the L-L3 cDNA, and the Renilla expression plasmid was always mixed in the stock solution that subsequently was used to dilute the Luciferase plasmids (L, L1, L2 and L3) to assure an equal basal Renilla expression level.

To investigate whether the conserved AREs in the 3′-UTR were destabilizing motifs, a series of mRNA stability experiments with Actinomycin D were performed. The half-life of endogenous Wnt-5a upon Actinomycin D treatment was ~3 h (data not shown). Importantly, the addition of the 3′-UTR regulatory domains to the Luciferase gene did not affect the stability of the mRNAs as shown by similar transfection experiments described above, and subsequent northern blotting using Luciferase and Renilla specific probes (Figure 2D). Indeed, treatment with Actinomycin D for 3 h did not lead to a decreased amount of mRNA indicating that the regulatory domains in the first 1–258 bp of the 3′-UTR were neither mRNA destabilizing motifs, nor stabilizing motifs. Therefore the data suggested that the evolutionarily conserved elements could suppress translation of the Wnt-5a gene.

HuR is one important protein that binds the Wnt-5a 3′-UTR AREs

To further characterize possible mRNA binding proteins that can bind the AREs in the Wnt-5a 3′-UTR we performed a UV-crosslinking experiment with radiolabelled mRNAs corresponding to the coding region of Wnt-5a as control (ctrl) or the different 3′-UTR fragments (1: 1–66, 2: 1–183, 3: 1–258 bp) together with cytoplasmic or nuclear extracts of HB2 cells. The reactions were subjected to a 10% SDS–PAGE and analysed by autoradiography. As shown in Figure 3A (left), there were at least four different proteins binding to the mRNAs. One protein of 20 kDa bound within the first 1–66 bp, one at 36 kDa within 66–183 bp and two (98 kDa and 45 kDa) showed enhanced binding with increasing length of the 3′-UTR. As a control, a UV-crosslink experiment using probe 3 alone (3 w/o prot), or a mutated probe 3 (Δ3) together with nuclear lysates from HB2 cells, was performed (Figure 3A, right). As can be seen, the mutated probe 3 showed a decreased binding to the proteins corresponding to 36 and 98 kDa whereas probe 3 alone (3 w/o prot) did not give rise to background bands. HuR has a molecular weight of 36 kDa and is known to bind AREs of all classes (31). In fact, HuR is one ARE-binding protein that was previously reported to be affected in breast cancer (33–36).

To determine the ability of HuR to bind to the Wnt-5a 3′-UTR, REMSAs (Figure 3B and C) were performed with the control probe (ctrl) or the different 3′-UTR probes. When cytoplasmic and nuclear protein extracts were analysed a band appeared with the 3′-UTR probes 2 and 3 (1–183 and 1–258 bp). This correlated with the UV-crosslink data (Figure 3A). To analyse whether this band contained HuR, we performed supershifts (Figure 3C) with a HuR specific antibody together with probe 3 (1–258 bp; left) or a control IgG (right). Addition of the HuR specific antibody led to the disappearance of the faster migrating band, with a shift seen as a darker shade at the slower migrating bands. A cold probe (C) corresponding to probe 3 (1–258 bp) competed for binding (Figure 3C) and probe 3 alone (3 w/o prot) did not give any background bands.

To assure that HuR indeed bound to the Wnt-5a 3′-UTR we performed a UV-crosslink immunoprecipitation assay (Figure 3D). In contrast to immunoprecipitation with the specific anti-HuR antibody, the control IgG neither pulled down HuR nor the digoxigenin labelled probe 3 (left).

HuR suppresses Wnt-5a translation in vivo

We next wanted to investigate a possible role for HuR regarding Wnt-5a translation in vivo and therefore transfected the human breast epithelial line cell, HB2, with an expression vector coding for wild-type (wt) HuR or a mutated HuR (M1), which is unable to bind mRNA (44), together with the different Luciferase-3′-UTR clones (Figure 4A). Indeed, the over-expressed wt HuR protein affected the L2 and L3 expression levels significantly as compared to the vectors that do not bind HuR (L and L1). In contrast, the mutated HuR plasmid M1, that cannot bind mRNAs, did not significantly affect any of the Luciferase-expression plasmids. Moreover, the Luciferase-3′-UTR mRNA levels remained constant even in the presence of over-expressed HuR, irrespectively of Actinomycin D treatment as indicated by northern blots (Figure 4B). Thus, this clearly suggests that HuR did not affect Wnt-5a mRNA stability when bound to the AREs of L2, but rather regulates the translation efficiency. The transcription rate of Luciferase in vitro was unaffected by addition of the 3′-UTR elements with exception for the L2 construct (discussed in detail below), as measured by incorporated [35S]methionine, corrected for (Figure 4C). The translation efficiency was also measured in a cell free system using Rabbit reticulocyte lysate together with the Transcend™ non-radioactive translation detection system. The protein content of the rabbit reticulocyte lysate system is to our knowledge not fully known. However, one can expect that the in vitro translation system differ from the cellular context regarding the function of various
Figure 2. (A) Schematic drawing of the eight constructs produced with HA-Wnt-5a (W) followed by 1–66 bp (W1), 1–183 bp (W2) and 1–258 bp (W3) of the Wnt-5a 3’-UTR; or Luciferase (L, L1, L2 and L3). (B) Western blot of transfected (W, W1, W2 and W3) HB2 human mammary epithelial cells revealed with anti-HA antibody to avoid endogenous Wnt-5a detection. The lower panel shows a histogram (mean ± SD) representing a ratio between OD measurements of HA-tag/OD endogenous Wnt-5a levels. (C) Luciferase assay of transfected (18–20 h) (L, L1, L2 and L3) HB2 human mammary epithelial cells cotransfected with Renilla. Relative Luciferase Units (RLU)/RLU (transfection efficiency control) indicate decreased expression of Luciferase reporters containing 3’-UTRs. Error bars indicate SEM (n = 12). (D) Transfections in (C) were treated with Actinomycin D for 3 h and subjected to northern blot with Luciferase and Renilla specific probes. The lower panel shows a histogram (mean ± SD) representing the ratio between OD Luciferase / OD Renilla. One out of four experiments is shown. Statistical analyses were performed using Student’s paired t-test (***P < 0.001).
mRNA binding proteins. We therefore performed one set of experiments using rabbit reticulocyte lysate only (Figure 4D upper panel) and one using HuR pre-programmed reticulocyte lysate (lower panel). We did not detect any significant difference regarding the in vitro translation efficiency between the Luciferase-3'-UTR constructs when using only reticulocyte lysate (upper panel) whereas translation of L3 was significantly inhibited when adding HuR pre-programmed reticulocyte lysate (lower panel). The fact that the L2 construct showed an increased transcription rate (Figure 4C), and that the L2 construct was less reduced than L3 in the previous transfection experiment...
Endogenous Wnt-5a protein levels in breast epithelial cell lines reflect Luc-3'-UTR levels

To ensure that not only protein levels (Figure 5B, upper panel) but also HuR localization (Figure 5A) were comparable, we performed immunohistochemistry with the HuR specific antibody. As shown in Figure 5A, HuR was primarily detected in the nuclei, with some cytoplasmic detection in all three cell lines tested; the non-cancerous HB2 cell line and the breast cancer cell lines MCF-7 and T47D. However, since these different human breast epithelial cell lines express varying protein levels of endogenous Wnt-5a (Figure 5B, lower panel), we wanted to analyse whether these expression levels reflected repression by HuR. Repression of the expression of transfected Luciferase-3'-UTR reporters should in theory reflect the ability and degree to which HuR could repress endogenous Wnt-5a levels in the respective cell lines.
Distinct from the variation in Wnt-5a protein expression between the cell lines, differences in expression are also evident following passaging and differ with the degree of confluency (45). Therefore in order to accurately represent genuine change in degree of HuR-mediated repression the cell lines tested, we ensured that cell lysates were consistently prepared from cells at similar stage of confluency (45). Therefore in order to accurately represent the genuine change in degree of HuR-mediated repression the cell lines tested, we ensured that cell lysates were consistently prepared from cells at similar stage of confluence. Transfection with the Luciferase-3'-UTR constructs indicated that repression of the Wnt-5a 3'-UTR reflected the endogenous levels of Wnt-5a expression in the respective cell lines. The construct carrying the potential HuR-binding site (L2) was most significantly affected indicating that increased repression of translation was evident where HuR had the ability to bind the 3'-UTR (Figure 5C). It is important to note that the modest differences obtained are probably explained by the difficulty in comparing totally different cell lines and the intrinsic variation of Wnt-5a, which we attempted to control for. Also, the fact that L3 is less affected than L1 in this experimental set up probably lies in the nature of the experiment as the two breast cancer cell lines used differ considerably in many aspects, including protein expression pattern. Nevertheless, taking these factors into account we can conclude that the reduction in the Luciferase expression seen in the experiment can be adequately explained by increased HuR-binding and may thus be indicative of the endogenous control of Wnt-5a translation.

Wnt-5a translation is further suppressed during hypoxia

Hypoxia occurs in solid tumours where circulation is compromised because of structurally disorganized blood vessels (46). During hypoxia, active HuR has been shown to translocate to the cytoplasm and protein expression of HuR was increased (26,32). To investigate in detail the effect of HuR on Wnt-5a translation under hypoxic conditions, we transfected HB2 cells with the Luciferase-3'-UTR constructs and placed one dish in a normoxic chamber and one in a hypoxic chamber for 24 h. Indeed, the translation efficiency of the constructs carrying HuR-binding sites were significantly (P < 0.01) suppressed under hypoxic as compared to normoxic conditions (Figure 6A), probably resembling the situation of increased HuR activity in invasive breast cancer as previously reported (35,36). A discrete increase in HuR levels was detected in hypoxic cells just as previously reported (32) (Figure 6B). Finally, the endogenous Wnt-5a mRNA and protein levels were analysed after culturing HB2 cells for 24 h under hypoxic, as compared to normoxic conditions. The Wnt-5a mRNA levels remained stable during hypoxia and did not decrease as judged by semi-quantitative RT–PCR (Figure 6C). If anything we observed a slight increase in endogenous Wnt-5a mRNA under hypoxic conditions. In the same samples, however, we found a consistent decrease in endogenous Wnt-5a protein expression under hypoxic conditions (P < 0.05; Figure 6D). The same experiments were repeated in MCF-7 cells and similar results were obtained (data not shown).

DISCUSSION

The importance of Wnt-5a signalling in metastasis has been a matter of debate since conflicting results concerning the expression levels of Wnt-5a in invasive tumours emerged in the literature (9,12,18–24). One of the major reasons for this is that most early studies concerning the role for Wnt-5a in cancer, examined Wnt-5a mRNA and not protein levels. We have previously shown that expression of the tumour suppressor, Wnt-5a, is lost or severely down-regulated in invasive human breast cancer, and hence is a predictor of longer disease-free survival (12,13). The lack of expression was not caused by a transcriptional defect since high amounts of Wnt-5a mRNA was detected in otherwise protein negative tumours (13). Wnt proteins have not generally been viewed as proteins that are regulated through a post-transcriptional mechanism. However, there are two reports concerning 3'-UTR post-transcriptional regulation of non-mammalian Xwnt-8, Awnt-5A, Awnt-5B and Awnt-1 (47,48). These studies...
indicate that the Wnts analysed are regulated by mRNA stabilization as well as mRNA localization. In the present study we present the novel finding that the human Wnt-5a mRNA contains evolutionarily conserved sequence elements (AREs) in its 3'-UTR that are implicated in post-transcriptional regulation. Moreover, a recent global analysis of HuR regulated genes identified Wnt-5b as a potential target (49). Wnt-5a and Wnt-5b are almost identical in their coding region, but their 3'-UTR are different. The 3'-UTR of Wnt-5b is neither conserved, nor does it contain a repetitive AU-rich stretch, as in the 3'-UTR of Wnt-5a. Here we show for the first time that Wnt-5a is regulated at the post-transcriptional level. The regulation is caused by a HuR-mediated suppression of translation. We further show that the low Wnt-5a protein expression level in breast tumours is not caused by HuR-mediated destabilization of the Wnt-5a mRNA. Rather, and in line with our previous findings (13), the mRNA levels in otherwise Wnt-5a protein negative breast cancers have a high or normal level of Wnt-5a mRNA.

Several RNA-binding proteins that are specific for AREs have been characterized (26,27). The Elav-like protein HuR has been previously reported to primarily stabilize mRNAs (31). We show that four proteins bind to the most conserved sequence elements of the human Wnt-5a 3'-UTR; 20, 36 (corresponding to HuR), 45 and 98 kDa. The data presented in this study strongly indicate that HuR binds to the 3'-UTR of Wnt-5a and, importantly, it binds AREs to suppress translation. The fact that Hu proteins can bind mRNAs to affect translation has been observed previously (29,50), although with distinct differences (i.e. increased translation or inhibition of IRES-dependent translation). An important similarity to our study however, is that the Hu proteins were also suggested to bind AREs of class III in these studies (29,50,51). Furthermore, our finding is supported by a recent study where HuR was reported as a negative translational regulator in mammalian cells acting in synergy with the translational repressor TIA-1 (30). TIA-1 and TIAR belong to the same family of proteins and are both translational repressors of ~45 kDa (25). We believe that HuR is an essential protein for regulating translational suppression, most likely in cooperation with at least one more protein as has previously been suggested (30,52). Invasive human breast cancers have high levels of HuR in the cytoplasm (HuRc) (36). Invasive human breast cancers also have low Wnt-5a expression levels (12,13). Clinical support for the concept that HuR acts in synergy with at least one more protein comes from the finding that an increased level of HuRc in invasive breast cancer only corresponds to tumour grade, whereas loss of Wnt-5a expression correlates to survival.

It has been shown that HuR affects mRNA and translation primarily in the cytoplasm, as exemplified by the increased Cox-2 mRNA and protein expression levels found in HuR(+), positive breast cancers (35). It is therefore possible that the decreased protein expression level of Wnt-5a in human invasive breast cancer also is a direct consequence of HuR expression and cellular distribution. Hypoxia that exists in many solid tumours, leads to an increased expression of HuR but also leads to translocation of active HuR to the cytoplasm (32). We have taken advantage of this finding to mimic the HuR phenotype in invasive breast cancers and consequently found a decreased Wnt-5a translation rate during hypoxic conditions as shown using both Luciferase reporter vectors and endogenous Wnt-5a protein. Whether hypoxia affects other RNA-binding proteins, in addition to HuR, is not known. Interestingly, TIAR has been reported to be upregulated in neurons after ischemic brain injury (53).
A growing number of genes that are affected by HuR, or related proteins, are reported. Many of them are genes associated with inflammation, but also with tumourigenesis and metastasis, such as c-myc, p53, VEGF, Cox-2, the cyclins (26) and now Wnt-5a. Whether the altered activity of HuR is caused by differentiation, hypoxia, nutrient-starvation or by other unrelated factors in the primary tumours will need to be further investigated. However, the fact that the effects of one group of proteins can affect numerous target proteins is intriguing both in the aspect of inflammation and as shown here, also in that of cancer. Efforts should be made to understand these mechanisms better with the goal to design novel cancer therapies based on transcript-based regulatory proteins.

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