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β-catenin-independent WNT signaling in basal-like breast cancer and brain metastasis

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A role of WNT signaling for primary breast cancers of the basal-like subtype and as a predictor of brain metastasis has been described. However, a responsible WNT ligand has not been identified. To further clarify this question, we comparatively investigated 22 human breast cancer brain metastases as well as the highly invasive human breast cancer cell line MDA-MB-231 and the weakly motile MCF-7 as models for the basal-like and the luminal A subtype. WNT5A and B were found overexpressed in MDA-MB-231 cells as compared with MCF-7. This corresponded to reduction of MDA-MB-231 invasiveness by WNT inhibitors, whereas MCF-7 invasion was enhanced by recombinant WNT5B and abolished by WNT and Jun-N-terminal kinase antagonists. Expression and subcellular distribution of β-catenin remained uninfluenced. Consistently, β-catenin was not localized in the nuclei of brain metastases while there was strong nuclear c-Jun staining. Similar to MDA-MB-231, metastases showed expression of WNT5A/B and the alternative WNT receptors ROR1 and 2. These findings were validated using external gene expression datasets (Gene Expression Omnibus) of different breast cancer subtypes and brain metastases. Hierarchical cluster analysis yielded a close relation between basal-like cancers and brain metastases. Gene set enrichment analyses confirmed WNT pathway enrichment not only in basal-like primaries but also in cerebral metastases of all subtypes. In conclusion, WNT signaling seems highly relevant for basal-like and other subtypes of breast cancers metastasizing into the brain. β-catenin-independent WNT signaling, presumably via ROR1-2, plays a major role in this context.

Introduction

Breast cancer is very heterogeneous. In microarray studies, five molecular subtypes have been identified by hierarchical clustering (1) correlating with morphology and patients' survival (2,3). The molecular luminal A and B subtypes correspond to the histologically hormone receptor-positive tumors, the ERBB2 cluster to the subentity with human epidermal growth factor receptor 2 (c-erbB2)-overexpression and the basal-like cohort to the so-called triple-negative (TN) breast cancers. The latter are associated with a particularly unfavorable clinical course not least because of frequent metastasis to the brain (4).

In an attempt to characterize specific signaling pathways, which determine the different biological course of the various subtypes and the preferential sites of distant organ involvement, Smid *et al.* (5) found members of the WNT signaling pathway overrepresented in the basal-like subtype as well as in primary breast cancers which later metastasized to the brain. However, no responsible WNT ligand could be identified.

The WNT pathway regulates central steps in embryonic development and adult tissue organization (6). When signaling via β-catenin, binding of a WNT ligand to a Frizzled receptor and its co-receptor low-density lipoprotein-related protein 5/6 induces recruitment of the mediator protein Disheveled. This leads to inhibition of a constitutively active β-catenin destruction complex, followed by accumulation and translocation of β-catenin into the nucleus. There, it functions as a transcriptional coactivator together with the transcription factors of the lymphoid enhancer factor and T-cell factor family (7). Apart from this, WNT signals can also be channeled through Disheveled into several β-catenin-independent pathways, such as the WNT/Ca^{2+} cascade (8), the planar cell polarity pathway (9), as well as the recently identified signaling branch via the receptor tyrosine kinase-like orphan receptors (RORs) 1 and 2 (10).

WNT signaling is involved in embryonic and adult breast development (11). While signaling via β-catenin is important in all antenatal phases of mammary gland development, in the postnatal prepubertal and pubertal gland organization predominantly WNT ligands with β-catenin-independent signaling potential, namely WNT 2, 4, 5A, 5B, 6 and 7B are found enriched in epithelia and/or stroma (12).

In breast cancer, WNT1/β-catenin signaling, driven by the mammary tumor virus long terminal repeat (MMTV) promotor, is crucial for malignant transformation in the MMTV-WNT1 mouse model (11). In contrast to colon cancer, where deregulated signaling via β-catenin is the crucial event (13), the significance of this pathway for human breast metastasis is still not clear. Equally, there is much controversy regarding the potential role of alternative ligands, such as WNT5A (14). It can be expressed directly in the epithelial tumor cells (15) but also in tumor-associated macrophages followed by enhanced cancer cell invasion via activation of WNT/β-catenin-independent signaling and the Jun-N-terminal kinase (JNK) (16).

To further clarify whether WNT signaling, in fact, is crucial for the biologically aggressive behavior of basal-like versus luminal breast cancers and their preference for specific target organs of metastasis, we chose the human breast cancer cell lines MDA-MB-231 and MCF-7. The former is highly invasive and has been demonstrated to correspond at the molecular level to the basal-like subtype, whereas the latter is only weakly invasive and closely related to the luminal A subtype (17,18).

In order to identify potential WNT ligands and their mode of signaling, these model cell lines were compared with regard to expression of WNT pathway members as well as to their functional response to the identified ligands and the respective inhibitors. The findings were further validated by hierarchical clustering and gene set enrichment analysis of three external datasets as well as in histological samples of 22 human breast cancer brain metastases. We could show that β-catenin-independent WNT signaling is critical for the biologically aggressive behavior of basal-like cancers and for other subtypes that metastasize to the brain. WNT5A and B as well as the receptors ROR1 and ROR2 are probable candidates that could mediate these signals and may be potential targets to influence progression of the unfavorable course of basal-like breast cancers and development of brain metastasis in general.

Material and methods

Cell lines, cell culture and tissues

MCF-7 and MDA-MB-231 were cultured in RPMI 1640 media supplemented with 10% fetal calf serum. For gene expression and protein studies, cells were seeded on extracellular matrix (R&D Systems, Wiesbaden, Germany)-coated

Abbreviations: DKK, Dickkopf; ER, estrogen receptor; GEO, Gene Expression Omnibus; JNK, Jun-N-terminal kinase; KEGG, Kyoto Encyclopedia of Genes and Genomes; ROR, receptor tyrosine kinase-like orphan receptor; sFRP, secreted Frizzled-Related Protein; qRT–PCR, quantitative real-time–polymerase chain reaction; 3D/2D, three-dimensional/two-dimensional; TN, triple-negative.

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tissue culture wells. Recombinant murine WNT5A, WNT5B and human Dickkopf (DKK)-1 and secreted Frizzled-Related Protein (sFRP)-1 were all obtained from R&D Systems. All patient samples were collected after informed consent during medically indicated neurosurgical procedures following approval by the local ethics committee.

RNA isolation, WNT array and quantitative real-time–polymerase chain reaction

RNA was isolated with Trizol (Invitrogen, Karlsruhe, Germany) followed by a DNase (Roche, Mannheim, Germany) digestion step. Complementary DNA synthesis, hybridization and chemiluminescence detection were performed with the 'WNT Signaling Pathway' GE Array Q Series (Superarray, Frederick, MD) according to the manufacturer's instructions. Reverse transcription was achieved with the iScript Master Mix (Bio-Rad, Munich, Germany). Quantitative real-time–polymerase chain reaction (qRT–PCR) was performed with messenger RNA-specific primers (supplementary Table 1 is available at *Carcinogenesis* Online) and the iTaqSYBRGreen + Rox Supermix (Bio-Rad) on the ABI PRISM 7900HT instrument (Applied Biosystems, Frankfurt, Germany). Gene expression was normalized to GNB2L1 and 18S ribosomal RNA.

Bioinformatics methods and statistical data analysis

All Affymetrix U133 Plus 2.0 datasets were retrieved from the NCBI Gene Expression Omnibus (GEO) data repository (19). After $\log_2$ transformation, the GSE10890 dataset containing normalized gene expression data of MCF-7 and MDA-MB-231 cells was analyzed for differentially expressed genes by fitting linear models separately for each gene using the empirical Bayes method (20).

Gene lists for relevant signaling pathways were obtained from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Genes were ranked according to the *P*-value for differential expression from the microarray experiments. In order to test for significantly enriched pathways, a one-sided Wilcoxon rank sum test was performed on the gene ranks for each pathway (21).

Three independent microarray datasets of 87 estrogen receptor (ER) positive (GSE6532), 18 basal-like tumor samples (GSE7904) and 15 brain metastases from primary breast cancers (GSE14020) (22) were used for cluster analysis. Raw data of all three datasets were preprocessed using Robust Multichip Average (23). After combining the single datasets, quantile normalization (24) was performed.

For survival analysis, additional 176 ER-positive breast cancer samples from Loi *et al.* were included, resulting in 263 ER-positive samples. 'Significance Analysis of Microarrays' (25) was performed to identify correlations between gene expressions and time to distant metastasis. To investigate if certain gene sets or pathways are overrepresented within the genes that show significant correlation between expression and metastasis development, overlaps between the gene lists were counted in contingency tables and significance was assessed using Fisher's exact test (26).

When necessary, *P*-values were adjusted for multiple testing using the method by Benjamini-Hochberg (27) and are subsequently called *q*-values. All analyses were performed using the free statistical software R (version 2.9.2, www.r-project.org) (28).

Invasion data and qRT–PCR expression data were compared with the unpaired two-tailed Student's *t*-test. *P*-values < 0.05 were considered significant.

Immunoblot and in vitro invasion assay

Immunoblots were performed as described before with the following monoclonal mouse antibodies: unphosphorylated β-catenin (Upstate, Temecula, CA) and E-Cadherin (BD Biosciences, Heidelberg, Germany) (16). Invasion of MCF-7 or MDA-MB-231 cells were measured in a modified Boyden chamber as described before (29).

Immunohistochemistry

To assess the receptor status, samples were routinely stained with anti-ERα, -progesterone receptor and -ERBB2 by the local Department of Neuropathology. Additionally, tissues were stained with either mouse monoclonal β-catenin antibody (Santa Cruz Biotechnology, Heidelberg, Germany) or rabbit monoclonal c-Jun antibody (Cell Signaling Technologies, Frankfurt am Main, Germany). β-catenin and c-Jun status and localization were independently evaluated by at least two researchers. To visualize morphology, samples were counterstained with hematoxylin.

Three-dimensional/two-dimensional migration assay

This assay was designed to circumvent the problem of unphysiological surfaces in the usual wound healing/scratch assays on plastic. It allows better assessment of the details of cell locomotion and, additionally, the simultaneous observation of two different cell types. Cells were seeded on matrigel-coated coverslips and grown to subconfluency. Two coverslips were turned upside-down and placed next to each other on a matrigel-coated cell culture dish [sandwich t/two-dimensional (3D/2D) culture]. Cells emigrating from under the coverslips form two fronts and migrate as cohorts toward each other (supplementary Figure 1 is available at *Carcinogenesis* Online). Before closure of the gap, the experiment was stopped. Cells were stained with Phalloidin-tetramethylrhodamine isothiocyanate (Sigma–Aldrich, Munich, Germany), mouse monoclonal E-Cadherin (Upstate), mouse monoclonal β-catenin (Santa Cruz Biotechnology) and rabbit monoclonal c-Jun (Cell Signaling Technologies). After the addition of Fluorescin isothiocyanate (FITC)-labeled secondary antibodies (Santa Cruz Biotechnology) and counterstaining with 4′,6-diamidino-2-phenylindole (DAPI, 1:1000, D8417; Sigma-Aldrich) and mounting (DakoCytomation, Glostrup, Denmark), slides were analyzed using a confocal laser scanning microscope (LSM 510; Zeiss, Göttingen, Germany).

Results

Identification of WNT5A and 5B in MDA-MB-231

First, we compared the WNT expression profile of the two model breast cancer cell lines. Using a WNT-specific complementary DNA array, we detected *wnt5b* and, to a lesser extent, *wnt5a* expression in the basal-like cell line MDA-MB-231, whereas no WNT ligand could be identified in MCF-7. *β-catenin* was expressed in a similar fashion in both cell lines, whereas there was a considerable difference in the signal intensities of target genes, such as *cd44*, *c-jun*, *fos like antigen 1* (FOSL1), *plasminogen activator*, *urokinase receptor* (PLAUR) and *vascular endothelial growth factor A* (VEGFA) (Figure 1A; supplementary Tables 2 and 3 are available at *Carcinogenesis* Online).

Array results were validated by qRT–PCR. This confirmed significant overexpression of *wnt5a*, *wnt5b* and *plaur* in MDA-MB-231, in contrast to *e-cadherin*, which was found overexpressed in MCF-7. *wnt3a*, *wnt10a* and the WNT/β-catenin target *axin2* showed no significant difference by qRT–PCR (Figure 1B). *wnt2* and *wnt7a* were undetectable. As an external validation, we compared our gene expression data with an NCBI GEO dataset of three independent MCF-7 and MDA-MB-231 samples (GSE10890). This confirmed overexpression of *wnt5a* and *plaur* in MDA-MB-231 and *e-cadherin* in MCF-7 (Figure 1C). Additionally, gene set enrichment analyses by Wilcoxon rank sum tests showed that, among others, the WNT pathway was differentially expressed between the two cell lines ($P < 0.001$) (supplementary Table 4 is available at *Carcinogenesis* Online).

Loss of E-Cadherin does not lead to β-catenin accumulation

E-Cadherin is known to bind β-catenin, thus, preventing its activation and nuclear translocation. As qRT–PCR revealed an enormous difference of *e-cadherin* expression between the cell lines, whereas *β-catenin* signal intensities in the complementary DNA array were similar, we were interested in whether the levels of unphosphorylated active β-catenin were different. Immunoblots with antibodies specifically recognizing the unphosphorylated form, however, detected higher amounts of activated β-catenin in MCF-7, even though these cells contain high levels of functional E-Cadherin (Figure 1D). Furthermore, immunofluorescence analysis with the same antibody revealed similar nuclear β-catenin levels in the investigated cell lines (data not shown). This indicates that the loss of E-Cadherin in MDA-MB-231 does not lead to accumulation of active β-catenin and, presumably, is not associated with upregulated WNT/β-catenin signaling.

Effects of WNT activation and inhibition on breast cancer cell invasion

We then asked whether the differentially expressed WNT ligands would explain the different biological behavior of the cell lines. In microinvasion assays, invasive capacity of the highly invasive basal-like MDA-MB-231 was reduced by addition of the physiological WNT inhibitors sFRP-1 and DKK-1 to 57 and 75%, respectively (Figure 2A; $P < 0.05$). Additional stimulation with exogenous recombinant (r) WNT5A and B could not further enhance MDA-MB-231 invasiveness (data not shown). In contrast, rWNT5B either alone

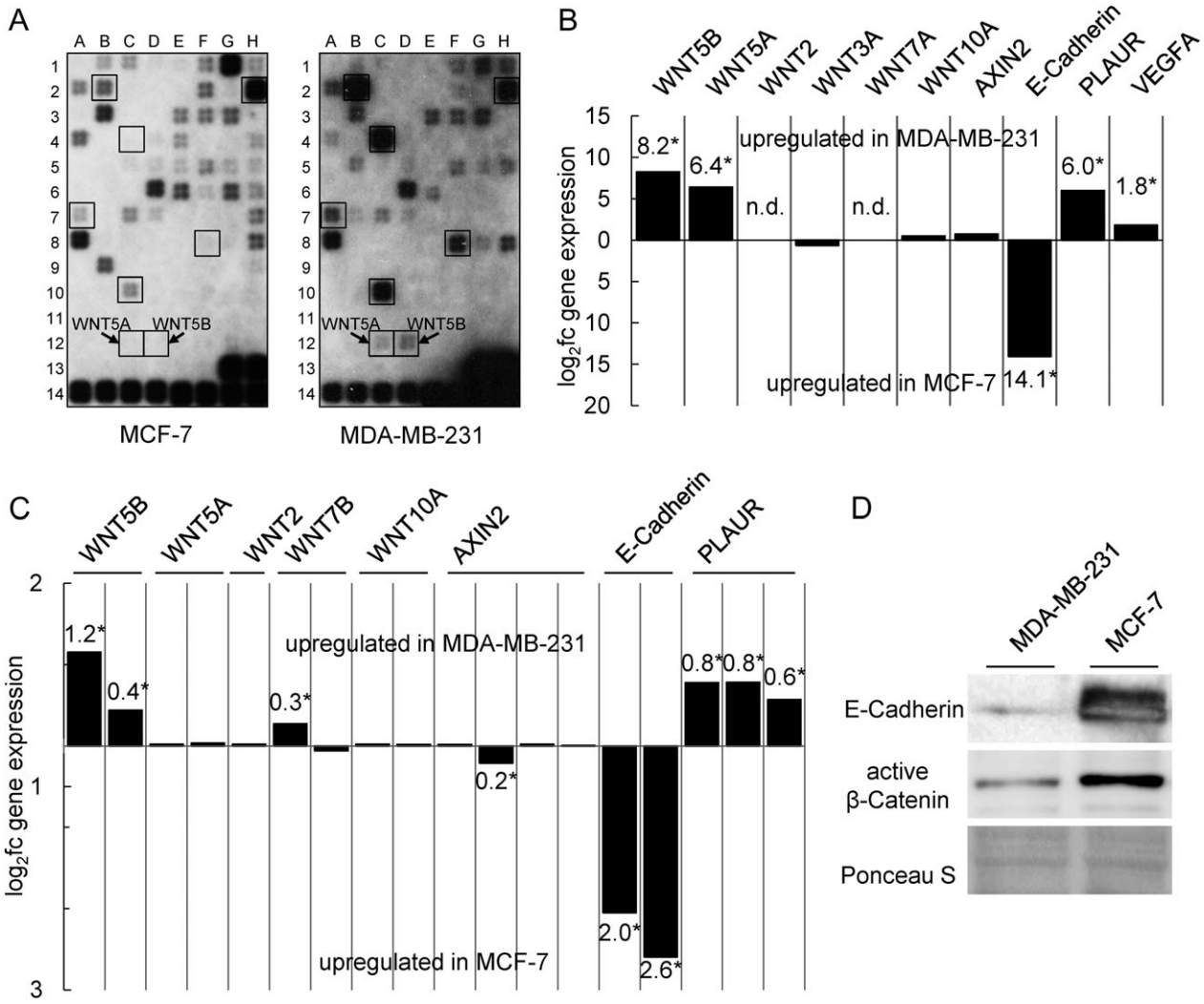

Fig. 1. (**A**) WNT signaling gene microarray assay of MDA-MB-231 and MCF-7 cells (for complete gene list and position, see supplementary Tables 1 and 2, available at *Carcinogenesis* Online). (**B**) qRT–PCR of MDA-MB-231 in comparison with MCF-7. Values shown are the $\log_2$ of the fold change calculated with the $\Delta\Delta CT$ method. (**C**) Gene expression analyses of the GSE10890 dataset. *y*-Axis shows the $\log_2$ of the fold changes of gene expressions. Asterisks represent significant expression changes; n.d., not detected. (**D**) Immunoblot for E-Cadherin and unphosphorylated β-catenin of MDA-MB-231 and MCF-7 whole-cell lysates.

or in combination with rWNT5A induced a 4-fold increase of invasion in MCF-7 (Figure 2B; $P < 0.05$). WNT5B-mediated invasion was counteracted by the inhibitors sFRP-1 and DKK-1 (both $P < 0.05$) (Figure 2C). We have shown previously that WNT5A-triggered invasion is mediated via JNK (16). Therefore, we focused on JNK signaling in WNT5B-induced invasion of MCF-7, which was significantly reduced by a JNK inhibitory peptide ($P < 0.05$) (Figure 2D). Neither stimulation of MCF-7 with WNT5A/5B nor inhibition of WNT signaling in MDA-MB-231 had any effect on the amount of unphosphorylated active β-catenin in both cell lines (Figure 2E–F). Likewise, nuclear extracts of MCF-7 cells showed no increase in active β-catenin upon stimulation with WNT5A/5B (data not shown). To ensure that the described effects on invasion where not due to changes in metabolism and viability, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide conversion was measured for all mentioned conditions yielding no differences (data not shown).

WNT expression in brain metastases

On the assumption that the identified WNT ligands influence the biological behavior of the two cell lines, we hypothesized that the WNT expression profile of human breast cancer brain metastases rather resembles the pattern of basal-like MDA-MB-231 than MCF-7. We therefore measured levels of *wnt3a*, *wnt5a* and *wnt5b* by qRT–PCR in six breast cancer brain metastasis samples of different histological subtypes (Figure 3A). Mean expression of each gene was then compared with the respective expression pattern of the MCF-7 breast cancer cell line. Results revealed a similar tendency as the differential gene expression between MCF-7 and MDA-MB-231. Expression of *wnt5a* and *wnt5b* was significantly stronger in the metastasis samples, whereas expression of *wnt3a* and *e-cadherin* was significantly lower. There was no difference for *axin2* and *plaur* expression (Figure 3B). The attempt to confirm expression of WNT5A/B protein in the brain metastases failed because of the lack of suitable antibodies.

To further clarify the role of alternative WNT signaling, we searched for possible receptors for WNT5A and WNT5B using the mentioned gene expression dataset of MCF-7 and MDA-MB-231 (GSE10890). This yielded significant upregulation of *ror1* ($q < 0.001$) in the basal-like cell line MDA-MB-231. There was no difference in expression of *ror2*. Expression of *ror1* and *2* was then investigated by qRT–PCR in the cell lines (Figure 3C) where we found a 237.3-fold upregulation of *ror1* in MDA-MB-231 as compared with MCF-7. There was no significant difference in *ror2* expression. In the breast cancer brain metastases samples (Figure 3D), the expression level of both *ror1* and *2* was above that of MCF-7 (fold change of 3.3 and 20.2, respectively).

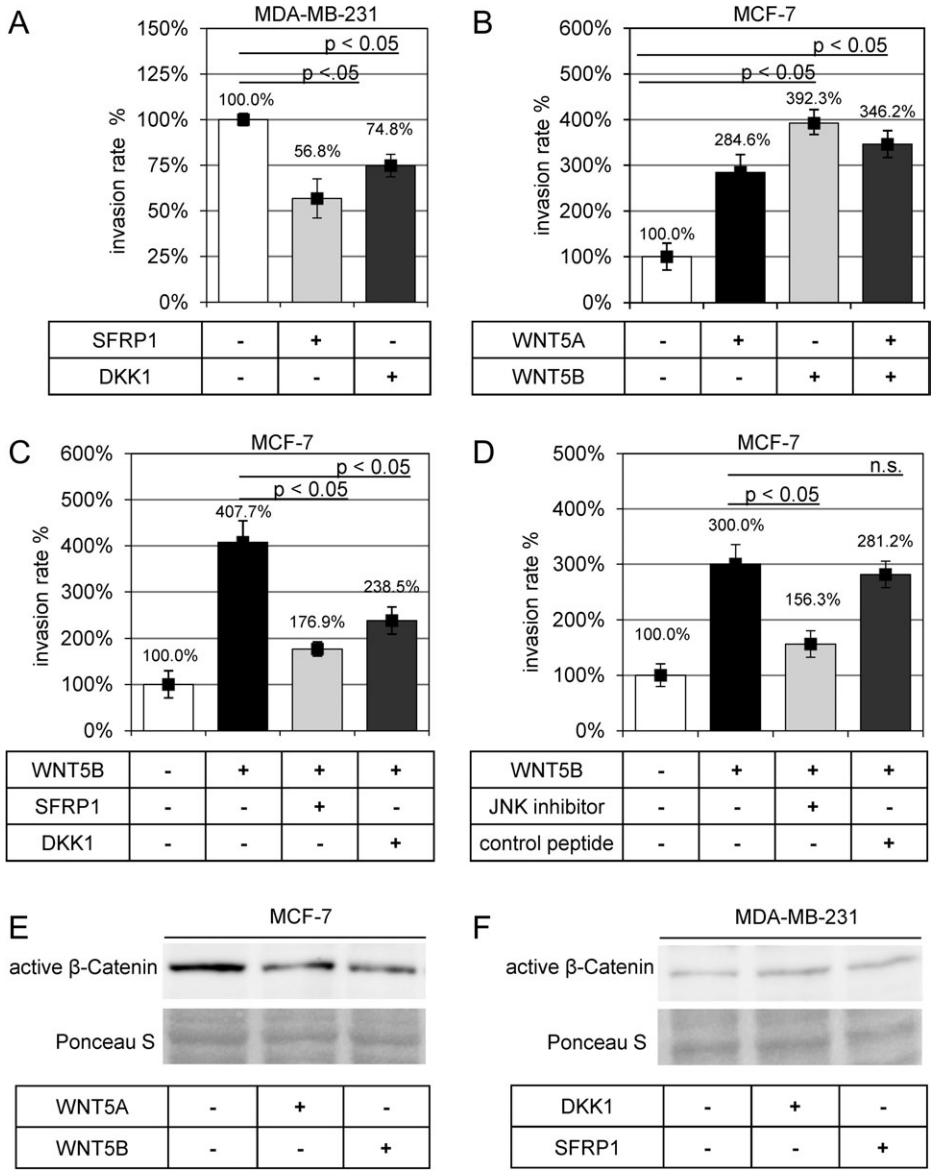

Fig. 2. *In vitro* microinvasion assay of (**A**) MDA-MB-231 in the presence of sFRP1 (400 ng/ml) and DKK1 (200 ng/ml), (**B**) MCF-7 cells with either WNT5A (100 ng/ml), WNT5B (100 ng/ml) or a combination of both WNTs (50 ng/ml each) added (**C**) MCF-7 with WNT5B and either sFRP1 (400 ng/ml) or DKK1 (200 ng/ml) and (**D**) MCF-7 cells with WNT5B and either the JNK-inhibitor SP600125 or the control peptide. Invasiveness is calculated as a percentage (means, ± SD, n = 4) of the untreated control. Immunoblot of unphosphorylated β-catenin of whole-cell lysates of (**E**) MCF-7 cells stimulated with either WNT5A (100 ng/ml) or WNT5B (100 ng/ml), (**F**) MDA-MB-231 treated with sFRP1 (400 ng/ml) and DKK1 (200 ng/ml); n.s., not significant.

To investigate the relevance of WNT/β-catenin and alternative WNT signaling for breast cancer brain metastasis, we stained 22 cerebral metastases samples for β-catenin and c-Jun. Colon cancer brain metastases with up to 100% nuclear β-catenin localization (data not shown) were used as controls. All breast cancer metastases were negative for nuclear β-catenin, except one sample of a TN cancer metastasis with a few positive nuclei (Figure 4A–C**;** supplementary Table 5 is available at *Carcinogenesis* Online). As nuclear β-catenin in colon cancer is detectable predominantly at the invasive front (30), we then specifically focused on this area. Sixty percentage of the 22 brain metastases also included adjacent benign tissue. However, even there, no nuclear β-catenin could be demonstrated. In contrast to the lack of nuclear β-catenin, the opposite was observed for c-Jun. A strong nuclear c-Jun signal was detectable in all of the breast cancer brain metastases (Figure 4D–E; supplementary Table 6 is available at *Carcinogenesis* Online). Taken together, this demonstrates that the WNT expression pattern of breast cancer brain metastases is close to that of the basal-like cell line MDA-MB-231. Upregulation is found for WNT/β-catenin-independent targets while there is no indication for activation of the WNT/β-catenin pathway.

β-catenin, E-Cadherin and c-Jun expression in migrating cells

To evaluate this observation on the functional level, we established a 3D/2D migration assay, where cells migrate on extracellular matrix toward each other. In contrast to the usual scratch or wound healing assays, cells move on a more physiological surface, thus, allowing a better assessment of the morphology of motile cells as well as the analysis of subcellular protein localization dependent on the cells' position in the migrating cohort. Furthermore, this method is suitable to directly compare two different kinds of cells in one cell culture well. The results of this assay underlined the above mentioned results. MCF-7 showed a much stronger β-catenin and E-Cadherin expression at the cell membrane as the more motile MDA-MB-231. None of the cell lines showed β-catenin accumulation in the cytosol or nuclei at the leading edge of migrating cohorts (Figure 5A–F; supplementary Figure 1 is available at *Carcinogenesis* Online). c-Jun was almost

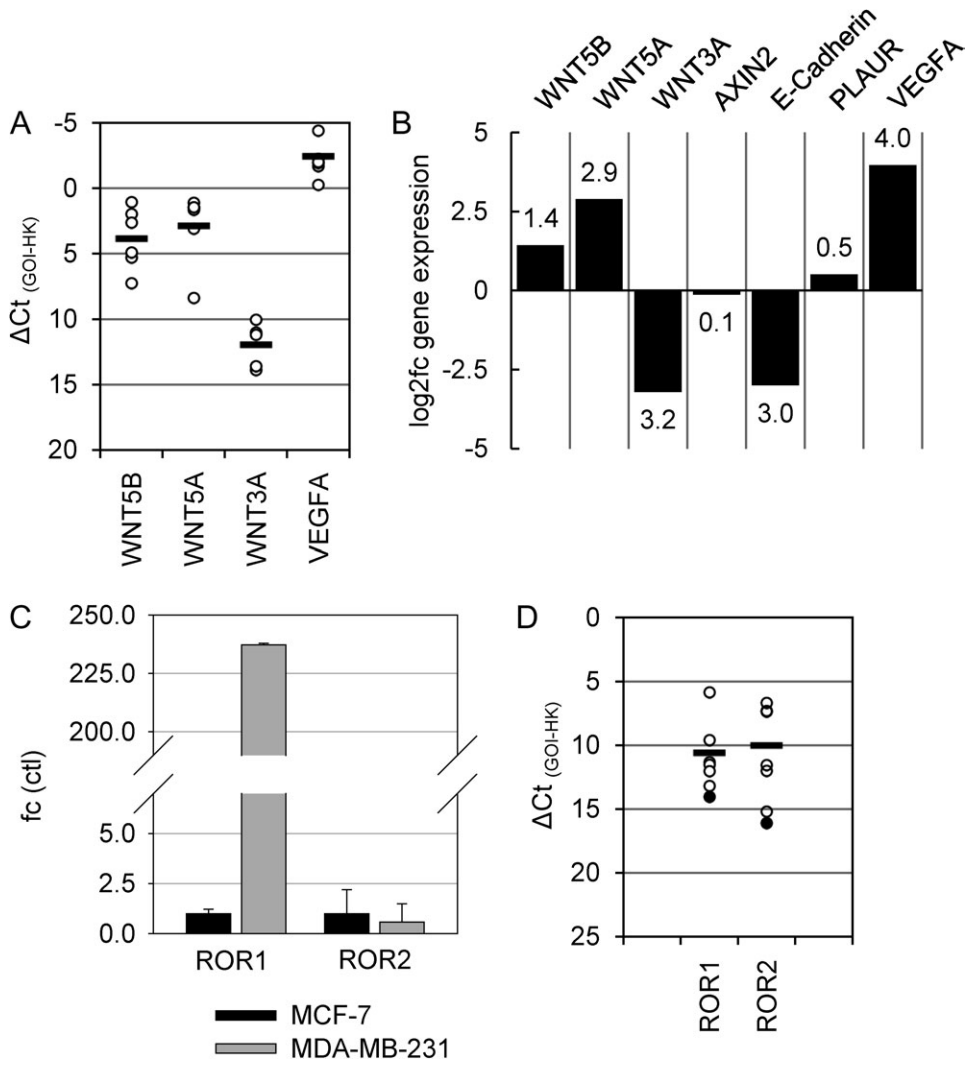

Fig. 3. (**A**) *wnt5b*, *wnt5a* and *wnt3a* messenger RNA expression levels in cerebral metastases of breast cancer patients. *vegfa* expression served as an internal positive control. Expression levels are calculated as the Δ*C*T between the gene of interest and the housekeeping genes. (**B**) Mean expression of *wnt5b*, *wnt5a*, *wnt3a*, *axin2*, *e-cadherin*, *plaur* and *vegfa* in the cerebral metastases in comparison with MCF-7 cells. Values shown are the $\log_2$ of the fold change calculated with the ΔΔ*C*T method. (**C**) qRT–PCR of *ror1* and *ror2* in MDA-MB-231 cells (gray bars) compared with MCF-7 (black bars). Values shown are fold changes calculated with the ΔΔ*C*T method. (**D**) *ror1* and *ror2* expression levels (Δ*C*T between the gene of interest and the housekeeping gene) as measured by qRT–PCR in cerebral metastases (open dots) of primary breast cancers. Filled dots show mean expression of MCF-7 cells.

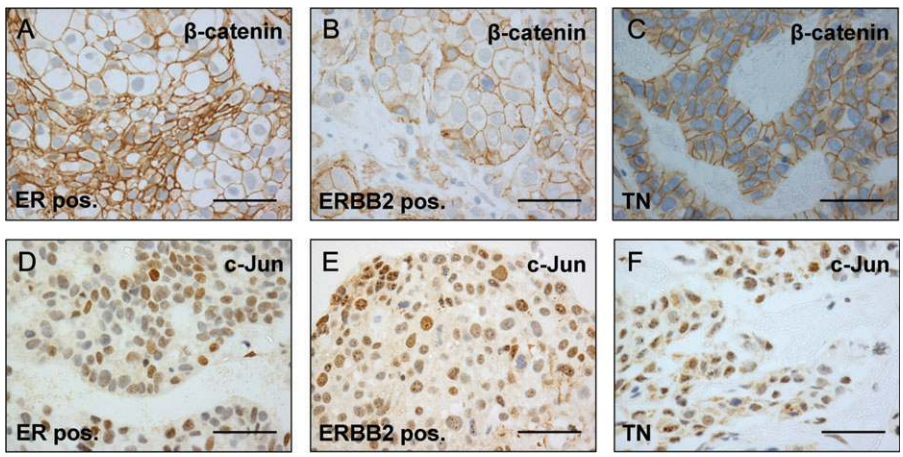

Fig. 4. β-catenin IHC staining of (**A**) ER positive, (**B**) ERBB2 positive and (**C**) TN cerebral metastases of primary breast cancers. c-Jun IHC of (**D**) ER positive, (**E**) ERBB2 positive and (**F**) TN cerebral metastases. Scale bar length of 20 μm.

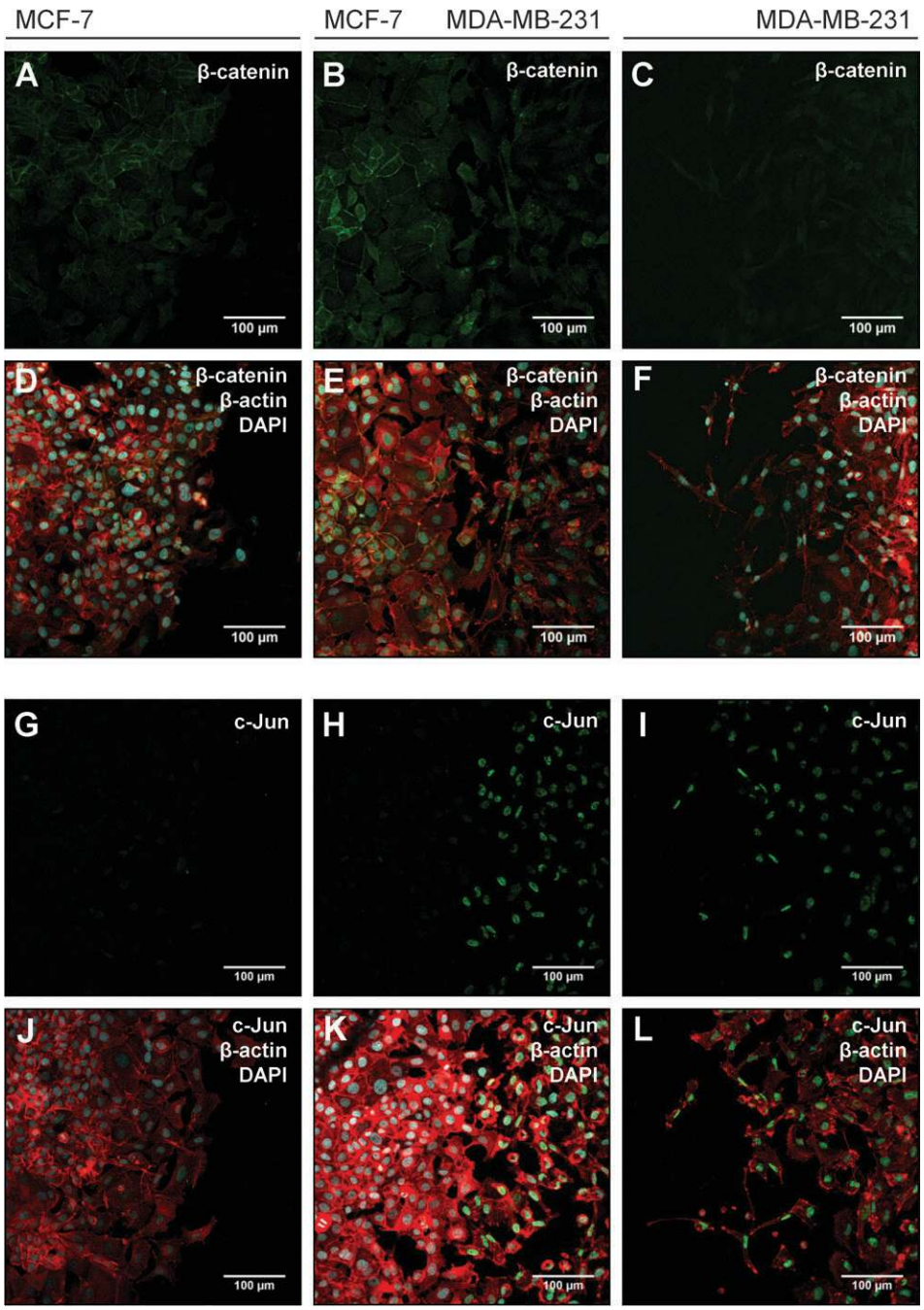

Fig. 5. Fluorescein isothiocyanate FITC-labeled β-catenin immunofluorescence staining of (**A**) MCF-7 cells, (**B**) MCF-7 (left) and MDA-MB-231 (right) and (**C**) MDA-MB-231 in a 3D/2D migration assay. FITC labeled c-Jun staining of (**G**) MCF-7, (**H**) MCF-7 (left) and MDA-MB-231 (right) and (I) MDA-MB-231. Cells were counterstained with tetramethylrhodamine isothiocyanate-labeled β-actin and 4′,6-diamidino-2-phenylindole (DAPI) (composite images **D-F** and **J-L**).

undetectable in MCF-7 but massively expressed in the nuclei of MDA-MB-231 independent of the cellular position within the motile population (Figure 5G–L).

Cluster analysis of primary breast cancer and brain metastasis datasets

To further characterize the impact of WNT signaling for the development of brain metastasis, we resorted to external datasets from the GEO database. Smid *et al.* (5) reported overrepresentation of the WNT pathway in primary breast cancer samples, which later metastasized to the brain. However, those analyses were performed in primary tumor samples and did not evaluate metastatic tissue. Hence, we scanned the GEO database for suitable datasets of luminal and basal-like breast cancer as well as for breast cancer brain metastasis data. This search identified three independent Affymetrix datasets: GSE14020, containing gene expression data of cerebral metastases (22), GSE6532, a dataset of hormone receptor-positive primary breast cancers (31,32) and GSE7904 with gene expression data of TN primaries (33). To allocate the brain metastasis samples to the various breast cancer subtypes, we evaluated the expression levels of estrogen, progesterone and ERBB2 receptors in GSE14020. All histological variants were present in the brain metastases dataset (supplementary Figure 2 is available at *Carcinogenesis* Online). We then performed normalization and hierarchical clustering of the three independent GEO datasets with the established gene set of Smid *et al.* (5). This yielded twoclusters,

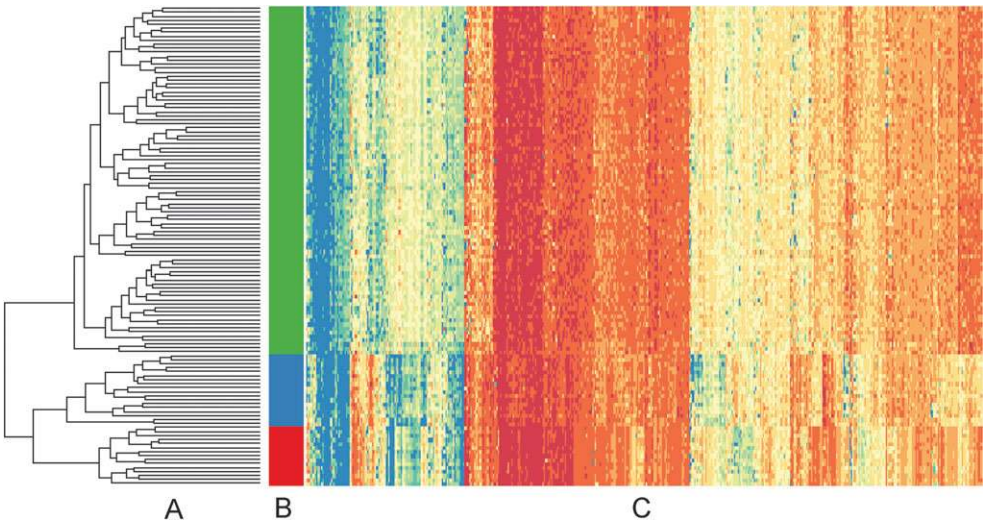

Fig. 6. Cluster analysis of ER positive, TN and brain metastases with heatmap of the relative gene expressions of the 'intrinsic' gene list by Smid *et al.* (genes in columns, tumor samples in rows). (**A**) The dendrogram indicates distances of the gene expressions between the tumor samples. (**B**) ER positive, TN and brain metastases are represented by the green, blue and red bar, respectively. (**C**) Blue color in the heatmap mirrors high gene expression, red low gene expression.

separating the ER-positive breast cancers in the first cluster from brain metastases and TN cancers in the second (Figure 6). Additionally, all cerebral metastases were individually clustered on a one by one basis into the cluster containing TN and the ER-positive cancers. Again, all metastases samples fell into the TN subcluster, independent of their histological subtype. As this method is based on the nearest neighbor principle, this indicates that the brain metastasis cluster is more closely related to TN breast cancers than to their respective original subtype.

Gene set enrichment analysis

Since our data point to a role of WNT signaling predominantly via β-catenin-independent cascades, we were interested in whether this could be corroborated by external datasets. The mentioned GEO dataset GSE6532 of 263 early stage hormone receptor-positive breast cancers (31,32) provides information on the incidence of distant metastasis and patients' survival. Performing Significance Analysis of Microarrays (25), we identified genes significantly associated with metastasis formation. By analyzing the correlation between gene expression and time to distant metastasis, we could show that the WNT pathway according to the KEGG derived gene set (WNT KEGG) was significantly overrepresented ($P = 0.0015$). This set comprises genes of each of the described WNT pathways, either dependent or independent of β-catenin. In contrast, gene sets containing exclusive information on genes signaling via WNT/β-catenin, such as the WNT3A (34) and the β-catenin gene set (35) were not significantly enriched ($P = 0.8296$ and $P = 0.2134$, respectively). This indicates that the WNT pathway is not only involved in dissemination into the brain but also in metastasis formation in general. Moreover, it strongly suggests that the WNT/β-catenin pathway most probably is not the pivotal WNT cascade in breast cancer metastasis.

Discussion

Metastasis is the major cause of mortality in breast cancer patients. The underlying mechanisms and signaling pathways are barely understood. Here, we report that WNT signaling is involved in this process and identify two potential WNT ligands in human brain metastases and a TN cell line. Most importantly, our results suggest that WNT signaling in tumor progression occurs independent of β-catenin — in contrast to WNT/β-catenin-mediated breast cancer initiation in various β-catenin mouse models (11).

Several clinical studies show deregulation of WNT genes (15) and physiological WNT inhibitors in primary breast cancers (36). However, the role of the WNT/β-catenin pathway is still inconclusive with regard to breast cancer progression and metastasis. While some authors demonstrated a prognostic impact of nuclear β-catenin (37), others found a prognostic value for reduced β-catenin (38) or suggested a ratio of membranous:cytoplasmic expression to predict outcome (39). Even a complete lack of predictive power of β-catenin has been described (40). Besides these contradictions, in the majority of the above mentioned studies nuclear localization of β-catenin was rare or even completely absent (41,42).

While all these investigations were performed in primary tumors, our study focused on β-catenin distribution in breast cancer metastasis, in particular, brain metastasis. There, nuclear β-catenin could be detected only in one of 22 cases. This is in line with the observation of weak or absent WNT/β-catenin reporter activity in breast cancer cell lines (43) and the rare frequency of β-catenin mutations in breast, in contrast to colon cancer (44).

On first glance, these data may challenge the notion of an essential involvement of WNT in breast cancer progression. However, other reports clearly indicate a role of WNT in this process (5,45). An obvious assumption is that WNT signals are conferred via β-catenin-independent pathways. This is supported by our finding that the highly invasive MDA-MB-231 and brain metastases have in common a strong expression of ROR1, WNT5A and WNT5B. Moreover, all of these proteins as well as ROR2 are overexpressed in brain metastases in comparison with the luminal A-like MCF-7. Since WNT5A and ROR2 are well-known mediators of β-catenin-independent WNT signaling, these results together with the demonstrated functional data strongly suggest a role of alternative WNT pathways in this setting.

We have demonstrated that WNT5A enhances invasion of the weakly invasive MCF-7 via JNK (16). Another study observed activation of the ERBB1 receptor by WNT5A (46). Interestingly, WNT1, usually signaling via β-catenin, can also activate ERBB1 in a β-catenin-independent way (47) and upregulate WNT5A. Along these lines, the WNT1 model shows only a modest AXIN2 activation in contrast to the ΔNβ-catenin tumor model without any differences regarding tumor progression (48). Thus, it stands to reason that at least some of the effects observed in the WNT1 mouse model are due to β-catenin-independent signaling (46,49). Consistently, we could not detect any difference of AXIN2 expression in MCF-7, MDA-MB-231 and the brain metastases,

which further suggest the involvement of β-catenin-independent signaling.

The ΔNβ-catenin mouse model consists mainly of epithelial cancer cells with a diminutive stromal compartment, whereas the WNT1-derived tumors recruit considerable amounts of stromal cells like fibroblasts and tumor-associated macrophages. We and others have shown that tumor-associated macrophage are a source for WNT5A and WNT5B (50,51). Here, we report that WNT5B, similar to WNT5A, induces invasion of MCF-7 cells, which can be reversed by sFRP1, DKK1 and JNK inhibition without any influence on β-catenin protein level and distribution. Although DKK1 has been originally considered an exclusive inhibitor of β-catenin-dependent WNT signaling, we and others have shown that DKK1 can exert its effects independent of β-catenin and can antagonize also non-canonical WNT signals (16,52). Interestingly, both WNT5 proteins are involved in terminal end bud differentiation of the adult breast, during which invasion of epithelial breast cells into the fat pad occurs. This invasion takes place without WNT/β-catenin reporter activity. Equally, in normal ductal elongation and branching WNT5A acts in a WNT/β-catenin-independent manner.

ROR1 and ROR2 have been identified as receptors for WNT5A (53–56). These homologous receptors seem to be involved in various kinds of malignancies, although little is known about the connection between ROR1-2 and WNT in breast cancer. The downstream effects of WNT/ROR1-2 activation are heterogeneous, the most consistent downstream target being JNK (57) and subsequently c-Jun. Our finding that WNT5A and WNT5B overexpression in basal-like MDA-MB-231 and brain metastases are accompanied by strong ROR1 expression suggests a common signaling pathway. The observation that WNT5A- and WNT5B-mediated invasiveness is antagonized by JNK inhibition fits into this pattern.

This is further supported by the fact that c-Jun, a major target of ROR signaling and JNK, showed strong nuclear expression in all the brain metastases, in contrast to β-catenin. Consistently, nuclear staining of c-Jun was much stronger in the highly invasive MDA-MB-231 than in MCF-7 in 3D/2D-migration assays. Recently, JNK was demonstrated to enhance invasion and induce partial epithelial mesenchymal transition in a basal-like cell line via c-Jun without any change of E-Cadherin and β-catenin (58). Histologies of breast cancers detected a correlation of c-Jun with the TN subtype (59) and demonstrated nuclear c-Jun predominantly at the invasive front (60). This may be explained by activation of c-Jun through the adjacent microenvironment. We recently reported that microglia, the resident macrophages of the brain, accumulate at the invasion front of brain metastases and are capable of inducing c-Jun in breast cancer cells. Microglia-assisted colonization of the brain by MCF-7 cells was dependent on active WNT signaling. This suggests that the susceptibility to c-Jun activation by WNT is an important prerequisite for breast cancer cells of any subtype to metastasize into the brain (51).

Analysis of external gene expression datasets further supports our hypothesis. We could show that brain metastasis is closely related to TN primary breast cancer, the histological correlative of the basal-like subtype where Smid *et al.* (5) originally identified overrepresentation of WNT signaling. In contrast to the WNT KEGG set, which was applied by Smid *et al.* and comprises all subtypes of WNT signaling, a recent study could not predict relapse in 368 primary breast cancers using gene sets of TCF4 and WNT3A signaling (34). Consistently, we could demonstrate that the WNT3A and β-catenin gene sets had no prognostic value in an ER-positive breast cancer data set, whereas the WNT KEGG set, containing members of alternative WNT signaling, still correlated with metastasis.

In conclusion, all these data confirm the significance of WNT signaling for breast cancer metastasis, especially into the brain. Based on the findings in colon cancer, most of the previous studies have focused on the classical WNT/β-catenin pathway without achieving clear-cut results. Our data rather suggest a role for the much less well-known alternative WNT receptors as well as for β-catenin-independent signaling in breast cancer progression.

Supplementary material

Supplementary Figures 1 and 2 and Tables 1–6 can be found at http://carcin.oxfordjournals.org/

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