ONCOLOGY REPORTS 17: 903-907, 2007

Expression of *Wnt5A* and *Wnt10B* in non-immortalized breast cancer cells

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Received October 23, 2006; Accepted November 7, 2006

Abstract. Wnt signaling is usually divided into two pathways: the 'canonical', acting through ß-catenin, and the 'non-canonical' acting through the Ca^{2+} and planar cell polarity pathway. Both pathways have been implicated in different types of cancer. Most results obtained with established cell lines have been contradictory. Here, we have investigated the expression of *Wnt10B* (canonical) and *Wnt5A* (non-canonical) in a panel of finite life-span and established normal and breast cancer cells using quantitative RT-PCR. It was found that there were both significant overexpression of *Wnt5*A and underexpression of *Wnt10B* in the metastasis-derived finite life-span breast cancer cells when they were compared to the finite life-span normal and established normal and breast tumor cells. Since expression profiles of primary breast cancer cultures are closer to the original tumor than the established cell lines, future research in this area should take into consideration these differences.

Introduction

Wnt proteins represent a large family of cell-secreted factors that control multiple aspects of early development in organisms ranging from invertebrates to mammals (1). The *Wnt* family consists of at least 19 highly conserved members that trigger different intracellular signaling pathways through either the 'canonical' or the 'non-canonical' pathway. In the canonical or ß-catenin pathway, *Wnt* ligands bind to two distinct families of cell surface receptors, the Frizzled (Fz) receptor family and the LDL receptor-related (LRP) family, and regulate the stability of ß-catenin, a key component of *Wnt* signaling. Once stabilized, the cytoplasmic protein ß-catenin associates with transcriptional factors of the LEF/TCF family and the TCF/ß-catenin complexes regulate expression of specific target genes. In the non-canonical pathway there are mainly two alternative ways of *Wnt* signaling which do not involve ß-catenin: the *Wnt*/Ca^{2+} pathway, which acts via calmodulin kinase II and protein kinase C, and the planar cell polarity pathway, which controls cytoskeletal rearrangements through Jun N-terminal kinase (2).

The role of *Wnt* genes in breast cancer has been studied since 1982, when the first *Wnt* member (called int-1) was identified as the gene activated by integration of the mouse mammary tumor virus, resulting in the development of mammary tumors in mice (3). Since then, an increasing number of studies have described an association between aberrant activation of the *Wnt* signaling pathway and human breast cancer (4-6). Nevertheless, the role of *Wnt* proteins in breast cancer has been controversial and not completely elucidated. While in colorectal cancer the aberrant *Wnt* expression is accompanied with known mutations in at least one component of the *Wnt*/ß-catenin pathway (7), in human breast cancer there are no reports of cancer-associated mutations in the *Wnt* genes (8,9). Moreover, the level of involvement of the different *Wnts* in the development and progression of human breast cancer is also a matter of uncertainty. While it can be hypothesized that overexpression for members of the canonical or ß-catenin pathway would stabilize ß-catenin, the role of members of the non-canonical pathway is more uncertain (10).

One such example is *Wnt5A*, which is associated with the non-canonical *Wnt*/Ca^{2+} pathway. It has mainly been considered as a tumor suppressor gene (11). There are conflicting reports in the literature showing both overexpression and underexpression of *Wnt5A* in breast cancer (12-15).

It is significant that most experiments related to the expression of *Wnt* genes in cell cultures have been performed with established cell lines. Array experiments have clearly demonstrated that there are important differences between the expression profiles of freshly finite life-span cultures and established cell lines (16-18); the finite life-span cell profile being more close to the original tumor.

To have a better insight into the *Wnt* expression in breast cancer cells, we have investigated the expression of *Wnt10B*,

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Key words: *Wnt5A*, *Wnt10B*, breast cancer

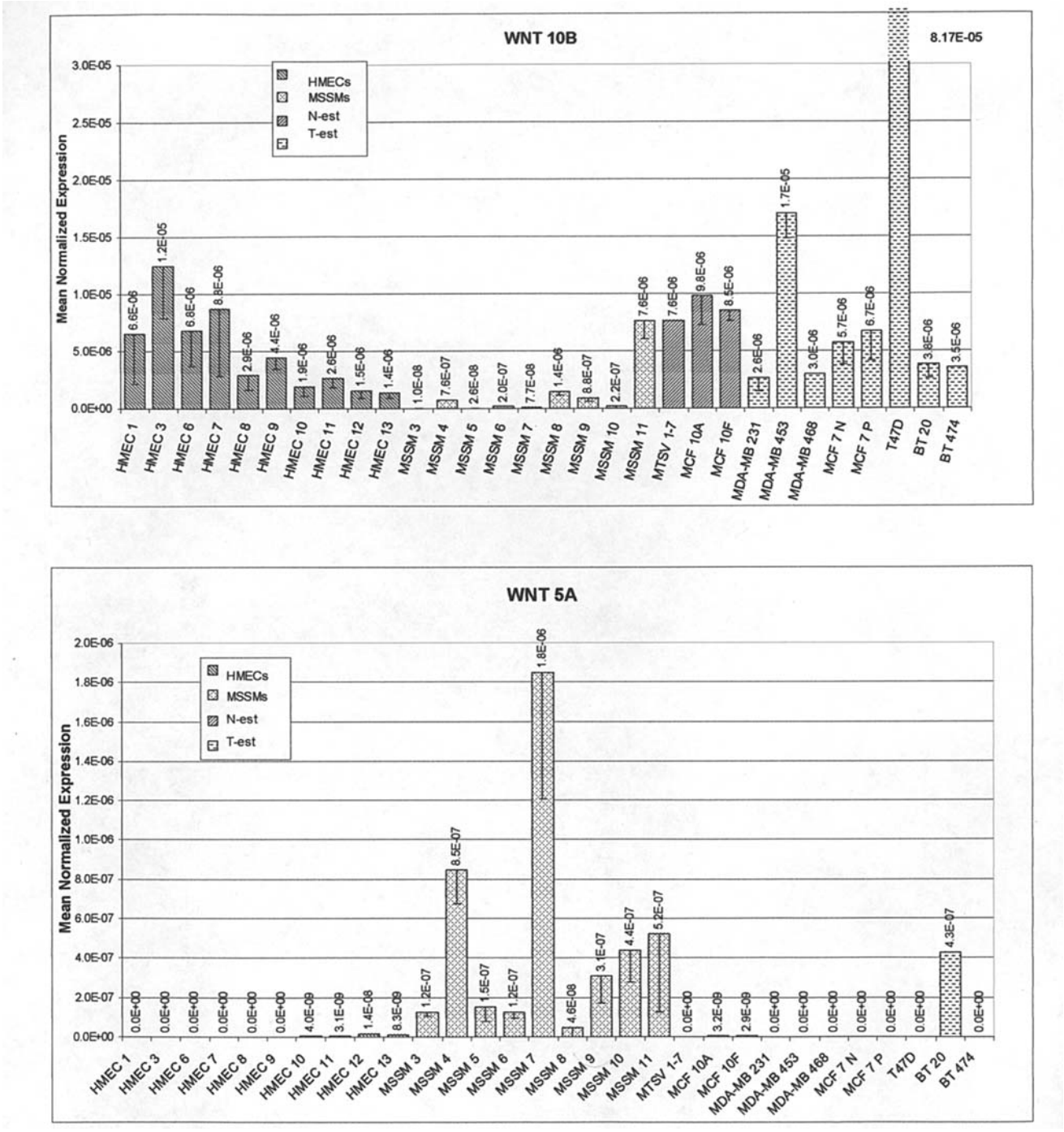

Figure 1. Expression of *Wnt5A* and *Wnt10B* in different cell populations. The mean and standard error of the normalized expression of the triplicates were calculated by Q-Gene. (A) Expression of *Wnt10B* in each cell line relative to G3PDH. (B) Expression of *Wnt5A* in each cell line relative to G3PDH.

a member of the canonical pathway, and *Wnt5A*, a member of the planar cell polarity (PCP) signaling pathway, in both, finite life-span and established normal and breast tumor cells by quantitative PCR.

Materials and methods

Cultures of finite life-span cells. HMECs, normal human mammary epithelial cell cultures (Biowhittaker Molecular Applications, Inc., NJ), were enumerated serially and maintained with MEGM (Clonetics, MD) supplemented with 2.5% FCS (Gibco Invitrogen, CA). MSSMs, breast cancer cells obtained from discarded ascitic fluids or pleural effusions of patients with metastatic breast cancer, were seeded and maintained in our laboratory. They were designated MSSM 3 through MSSM 11 and maintained with MEGM supplemented with 2.5% of FCS and 2.5% of the corresponding original fluid when available. For all the experiments, cultures of finite life-span cells, HMECs and MSSMs, were harvested in the 5th or 6th passage after initial plating.

Cultures of established cell lines. Normal-established (N-est): cell lines derived from normal tissues included MTSV1-7 (a gift from J. Taylor-Papadimitriou), MCF10A (from R. Mira y

Lopez) and MCF10F (ATCC, MD). Tumor-established (T-est) cell lines established from breast cancer pleural effusions included MDA-MB231, MDA-MB453, MDA-MB468, two strains of MCF7 (designated MCF7N and MCF7P) and T47D (ATCC, MD); and those from tumor tissues included BT20 and BT474 (ATCC, MD). They were maintained with the medium and supplements recommended by ATCC. All cell cultures, finite life-span and established, were harvested after 48-72 h of plating at about 80% confluence.

Quantitative real-time PCR (Q-PCR). RNAs were extracted with the Atlas pure total-RNA labeling system (Clontech, CA) according to the manufacturer's instructions.

Five micrograms of total-RNA was reverse transcribed with oligo(dT) (SuperScript II system, Invitrogen, CA) in a 20-μl reaction, and after a 125-fold dilution 40 pg cDNA (1.25 μl of the dilution) were used for PCR (40 cycles) on an ABI PRISM 7900 thermal cycler. The reaction was carried out in a 384-well plate with a QuantiTect SYBR-Green PCR kit (Qiagen Inc., CA) at an annealing temperature of 63˚C and detection at 77˚C (2-5˚C below the Tm of the product as determined by its dissociation curve). Product size was confirmed by agarose gel electrophoresis and identity of the amplified product was evaluated by blotting and hybridization with a specific probe and sequencing (data not shown). The efficiency of each pair of primers for amplification was determined, and expression of each gene relative to G3PDH was assessed by the program Q-Gene (19). Primers were designed using the program PrimerQuest (www.idtdna.com) or primer 3, unless otherwise stated. Primer sequences and lengths of products were: G3PDH-F, GTGAAGGTCGGAG TCAACGGA; G3PDH-3, GGTGAAGACGCCAGTGGACTC (300 bp) (20); *Wnt10B*-5, GCCATCCTCAAGCGCGGTTTC; *Wnt10B*-3, GAATCCAAGAAATCCCGAGAG (326 bp) (21); *Wnt10B* probe, CTGTGGCTGGAAGGGCAGTG; *Wnt5A*F, GGGGAAATGTGGTTTAATGGTG; and *Wnt5A*R, AAAT GGAGGTTGGAGACAAAGG (368 bp).

Statistical analyses. Since data were not normally distributed (many 0's and a few very large values relative to the other observations), the data summaries for each group are expressed as medians (and ranges) instead of means (and standard deviations).

All statistical comparisons between groups of the real-time PCR results were performed using the Wilcoxon-Mann-Whitney exact test using the StatXact (version 4) program. The $p<0.05$ level was used to determine statistical significance. All statistical tests were two-sided.

Results

We have explored the expression of *Wnt10B* (canonical pathway) and *Wnt5A* (PCP pathway) in nineteen finite life-span breast cell cultures and eleven established breast cell lines by real-time cDNA PCR. Mean expression values were normalized with the housekeeping gene G3PDH.

As shown in Fig. 1A, the immortal breast cancer cell line T47D strongly expressed *Wnt10B* while the other T-est lines showed a moderate expression similar to the N-est. Meanwhile, the HMECs showed a certain degree of variance among themselves. All of the MSSM cells, with the exception of MSSM11, showed clear down-regulation.

This down-regulation of *Wnt10B* in the finite life-span tumor cells is deemed statistically significant when compared with any of the other three classes of cells (Table I).

In contrast, as seen in Fig. 1B, expression of *Wnt5A* is only detected in the BT20 cell line within the T-est group as was previously reported (22). However, when we evaluated the normal cultures, only four out of ten HMECs showed a low level of expression similar to that of the N-est, while *Wnt5A* was undetectable in the other six. Remarkably, the expression of *Wnt5A* in the MSSM group of cells is statistically significantly higher than in the other groups (Table I).

Table I. Expression of *Wnt10B* and *Wnt5A* by cell class group.

Group (n)		Median (range)
Wnt10B		
HMEC (n=10)		3.65E-6 (1.36E-6-1.25E-5)
MSSM (n=9)		2.19E-7 (1.04E-8-7.64E-6)
N-est (n=3)		8.51E-6 (7.64E-6-9.81E-6)
T-est (n=8)		4.79E-6 (2.60E-6-8.17E-5)
MSSM vs HMEC	p=0.002[a]	
MSSM vs N-est	p=0.014[a]	
MSSM vs T-est	p=0.003[a]	
HMEC vs N-est	p=0.11 NS	
HMEC vs T-est	p=0.32 NS	
N-est vs T-est	p=0.28 NS	
Group (n)		**Median (range)**
Wnt5A		
HMEC (n=10)		0 (0-1.37E-8)
MSSM (n=9)		3.07E-7 (4.61E-8-1.85E-6)
N-est (n=3)		2.90E-9 (0-3.18E-9)
T-est (n=8)		0 (0-4.28E-7)
MSSM vs HMEC	p=0.0001[b]	
MSSM vs N-est	p=0.009[b]	
MSSM vs T-est	p=0.001[b]	
HMEC vs N-est	p=0.90 NS	
HMEC vs T-est	p=0.37 NS	
N-est vs T-est	p=0.15 NS	

[a]For *Wnt10B*, MSSM was significantly lower than the other three groups. [b]For *Wnt5A*, MSSM was significantly higher than the other three groups.

Discussion

Previous studies on the expression of *Wnt* genes in cultured cells were mainly performed with established cell lines, whose expression profiles may not represent the original tumor (16,23). The results shown here were obtained using a variety

of established and finite life-span normal and breast cancer cell lines.

Finite life-span cultures have the advantage of being closer to the original tissue than the established cell lines which accumulate mutations through repeated sub-culture passaging.

Our results showing up-regulation for *Wnt10B* expression in some of the T-est cell lines are in agreement with those obtained by Benhaj *et al* (22) who used non-quantitative RT-PCR. However, while their telomerase-immortalized HMEC shows a weak expression, our set of non-immortalized HMECs show a wide range of values, and so, when taken as a group, there is no statistical difference between them and the T-est group. Most significantly we have shown that *Wnt10B* is down-regulated in the MSSM finite life-span metastatic breast cancer cell lines compared to finite life-span HMECs and to metastatic T-est cell lines.

Wnt10B has long been considered an oncogene in mouse. However, in humans Bui *et al* (24) showed that it was expressed in some breast cancer lines but only in 3 of 50 breast tumors. No evidence for amplification or rearrangement of the gene was found. It was then concluded that *Wnt10B* may contribute to the malignant phenotype in a minority of breast cancers. Recently Milanovic *et al* (25) using *in situ* antisense RNA hybridization found that *Wnt10B* was detected at low levels in both normal and malignant breast tissues.

Our results from quantitative RNA expression analysis clearly show that *Wnt10B* expression is low in the finite life-span cells. They are in agreement with the results observed using breast tumors (25).

There are also contradictory reports in the literature about *Wnt5A* expression (26). Our results with the finite life-span breast cancer cell line clearly demonstrate that *Wnt5A* was up-regulated in these cells, but it was down-regulated in the T-est cells with one exception, the BT20 cell line.

These results agree with those obtained by Benhaj *et al* (22) regarding down-regulation in T-est cell lines. Nevertheless, in the MSSM finite life-span breast cancer cells, which are metastatic, we have found up-regulation at the level of mRNA expression in agreement with previous reports from studies in breast cancer (14,15) and other cancers (26). Although Dejmek *et al* showed reduction in protein *Wnt5A* expression in half of the invasive breast tumors studied, the same authors also demonstrated that *Wnt5A* RNA was expressed in those tumors when they performed *in situ* hybridization with a specific probe (12). *Wnt5A* acts through the non-canonical PCP signaling pathway. Its aberrant expression leads to more malignant phenotypes (abnormal tissue polarity, invasion, metastasis). Our results with metastasis-derived breast cancer cells fulfilled the expectation of activated *Wnt5* expression.

In summary, both *Wnt10B* and *Wnt5A* showed a significant differential expression between finite life-span and established breast cancer cells. Results from cDNA arrays have demonstrated that there are consistent differences between the expression profiles of finite life-span and established cell lines (16,18). Conclusions about breast tumorigenesis based only on the molecular biology of long established breast cancer cell lines may not represent the original tumor biology as well as freshly isolated cell lines. It has been shown that expression profiles of primary cultures are closer to the original tumor (16,18). Future research in this area should take into consideration these differences before reaching any definite conclusion.

Acknowledgements

This study was supported by grants from the T.J. Martell Foundation for Leukemia, Cancer and AIDS Research, the Jane Grinberg Memorial Fund, the Kash Family Foundation, and the Ellen Block Memorial Fund. We thank Christina De Pasquale for technical assistance and Dr Stella Melana for some of the finite life-span cells.

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