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Modification of Epigenetic Changes in Cancer by the Stromal Environment

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The stromal environment can modulate important epigenetic changes that are seen in carcinogenesis. We hypothesize that growth of primary, normal (non-tumorigenic) epithelial cells in the presence of stromal fibroblasts that have been isolated from a mammary adenocarcinoma will change the frequency of methylation events at promoter sequences such as for 14-3-3σ and p16. Promoter hypermethylation of 14-3-3σ is a frequent event in both early and later stages of breast cancer and silencing occurs in over 90% of breast tumors. We have successfully established a methylation-specific PCR assay to distinguish methylated from unmethylated alleles of the 14-3-3σ promoter. We found that 14-3-3σ is unmethylated in normal HMEC. We further determined that vHMEC, postulated to be a very early step in breast carcinogenesis, also lacks appreciable hypermethylation of 14-3-3σ promoter sequences. Interestingly, 14-3-3σ is methylated in mammary fibroblasts. To address whether mammary stroma affects the frequency of methylation in mammary epithelial cells using our established methylation-specific PCR assay, will require partitioning techniques to separate fibroblasts (with methylated 14-3-3σ sequences) from rare epithelial cells that may have hypermethylated the same sequences.

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Modification of Epigenetic Changes in Cancer by the Stromal Environment

DAMD17-03-1-0663

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INTRODUCTION

Concept: The stromal environment can modulate the important epigenetic changes that are seen in carcinogenesis. We hypothesize that growth of primary, normal (non-tumorigenic) epithelial cells in the presence of stromal fibroblasts that have been isolated from a mammary adenocarcinoma will change the frequency of methylation events at promoter sequences such as for 14-3-3σ and p16. Promoter hypermethylation of 14-3-3σ is a frequent event in both early and later stages of breast cancer and silencing of occurs in over 90% of breast tumors (Ferguson et al. 2000; Umbricht et al. 2001).

BODY

Specific aim (a) To determine the frequency of 14-3-3σ promoter methylation in human mammary epithelial cells that are grown in tissue culture in 2 dimensions (on plastic)

In order to determine the frequency of 14-3-3σ promoter methylation in human mammary epithelial cells grown in 2 dimensions, we cultured both HMEC and vHMEC (Romanov et al 2001; Hammond et al 1984) on plastic in MEGM medium and harvested 1-2x10^6 cells. DNA was extracted with a DNA extraction kit (Promega) and 1-2μg of genomic DNA was bisulfite modified as described (Herman et al 1996). Conversion was optimized in our hands such that an incubation with bisulfite at 55 C for 5 hours was used. We then carried out methylation-specific PCR on bisulfite treated DNA from both HMEC and vHMEC. Primers were designed as described (Ferguson et al 2000) to amplify either methylated or unmethylated alleles of the 14-3-3σ promoter. The conditions that gave specificity for methylated and unmethylated alleles were 95 C for 5 minutes followed by 31 cycles of 95 C for 30 seconds, 56 C for 30 seconds, 72 C for 30 seconds, followed by an extension cycle of 72 C for 4 minutes. Bisulfite modified DNA from the cell lines MDA-MB-231 and MDA-MB-435 that contain only unmethylated or methylated alleles was used as an unmethylated or methylated control respectively. DNA from human mammary fibroblasts was also obtained and tested.

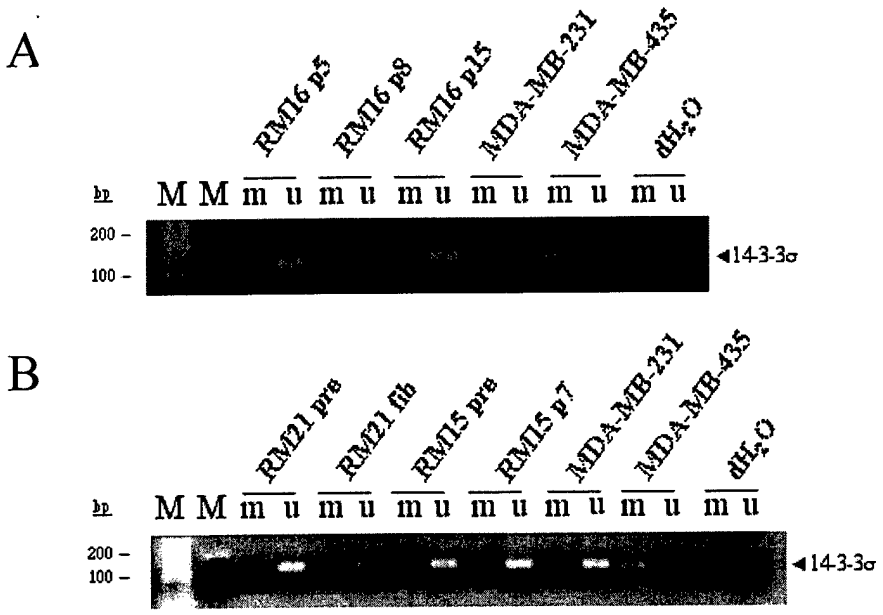

Fig.1: Methylation-specific PCR at the 14-3-3σ promoter in cells grown in 2-D. (A) Assessment of methylated (m) and unmethylated (u) alleles from vHMEC early (p5), middle (p8) and late (p15) passage of RM16. (B) Assessment of methylated (m) and unmethylated (u) alleles from HMEC (RM21 pre), associated mammary fibroblasts (RM21 fib), HMEC (RM15 pre) and vHMEC (RM15 p7). MDA-MB-231 and MDA-MD-435 were unmethylated and methylated controls respectively. Marker (M) lanes are indicated.

As expected, bisulfite modified DNA from MDA-MB-231 cells only gave products with unmethylated primer sets, while bisulfite modified DNA from MDA-MB-435 cells only gave products with methylated primer sets (Fig. 1) confirming the specificity of the assay. Bisulfite modified DNA from HMEC (RM15 and RM21) only gave products with unmethylated primer sets (Fig. 1). Bisulfite modified DNA from vHMEC (RM16 p5, RM16 p8, RM16 p15, RM15 p7) also only gave products with unmethylated primer sets. Interestingly, mammary fibroblast bisulfite modified DNA (RM21 Fib) gave products with both unmethylated and methylated primer sets. This may be due to hemimethylation in the fibroblast cells or complete methylation of a subpopulation of cells. Experiments are ongoing to determine the detectable frequency of methylated alleles in this assay. Thus, we found that vHMEC grown in 2 dimensions (on plastic) have unmethylated alleles of 14-3-3σ as detected by methylation-specific PCR.

Since one function of 14-3-3σ is to prevent a cell from entering G2 after its DNA has been damaged, we wanted to optimize DNA damage conditions that would arrest almost all vHMEC in the G2 phase of the cell cycle, so we could use this as a tool to enrich for those cells which escape the G2 arrest and assay this subpopulation for methylation of 14-3-3σ. A γ-irradiation procedure was established and HMEC/ vHMEC were grown on plastic for 48 hrs. At 48 hrs the cultures were irradiated with either 1Gy,

2Gy, 4Gy or 10Gy of γ-irradiation and maintained for an additional 24 hrs. BrdU was added for the final 4 hrs and the cells analysed by flow cytometry to assess their cell cycle profile.

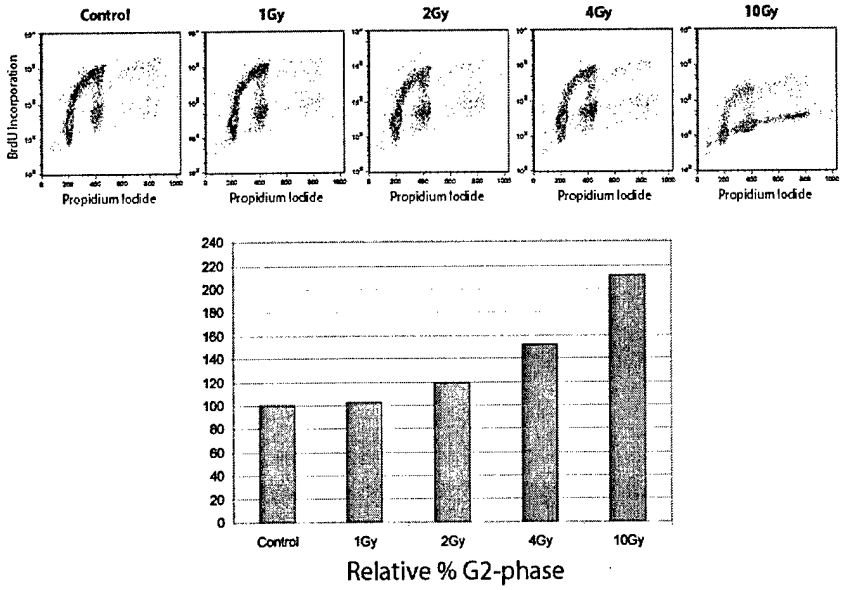

Figure 2: γ-irradiation induces a dose-dependent cell cycle arrest in adherent vHMEC. Variant HMEC were grown on plastic for 48 hrs. At 48 hrs the cultures were irradiated with the indicated dose of γ-irradiation and maintained for an additional 24 hrs. BrdU was added for the final 4 hrs. The cells were then collected and stained for BrdU and PI, and cell cycle fractions determined.

We found that γ-irradiation induces a dose-dependent G2 arrest. However, a substantial fraction of the cells were still cycling (Fig. 2). Even when the dose was increased to 10Gy, there remained a large fraction of cycling cells that escaped a G2 arrest. When irradiated cells were followed at later timepoints, the proportion of cycling cells increased (data not shown). This could be due to cells re-entering into the cell cycle or a reflection of the growth of cells that had never arrested. We had already shown that we could not detect methylation by PCR in vHMEC; although methylation of a subpopulation of cells within the vHMEC sample may not have been detectable with only a single round of PCR. Therefore, γ-irradiation was not a suitable method to induce a tight G2 arrest in vHMEC.

In order to optimize a 14-3-3σ dependent G2 arrest caused upon DNA damage, we chose to investigate chemicals that cause DNA damage and have been reported to arrest cells in G2. We focused on treatment of cells with nocodazole or doxorubicin (adriamycin). A nocodazole treatment procedure was established and vHMEC were treated with 100ng/ml nocodazole continuously for 24, 48 or 72hrs. BrdU was added for the final 4 hrs and the cells analysed by flow cytometry to assess their cell cycle profile. We found that nocodazole induces a cell cycle arrest in vHMEC similar to that seen in vHMEC treated with 10Gy γ-radiation (Fig.3). Again, although there was an increase in the percentage of cells in G2/M there was only a partial G2 arrest. Therefore, nocodazole treatment was not a suitable method to induce a tight G2 arrest in vHMEC.

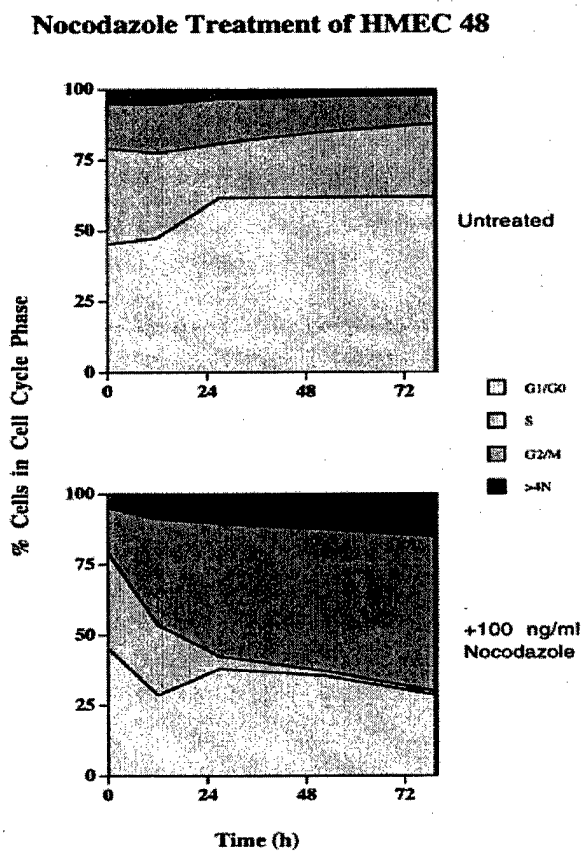

Figure 3: Nocodazole induces a cell cycle arrest in adherent vHMEC. Variant HMEC were grown on plastic and nocodazole (100ng/ml) was added 24, 48 or 72 hrs prior to harvesting. BrdU was added for the final 4 hrs. The cells were then collected and stained for BrdU and PI, and cell cycle fractions determined.

Another set of experiments was performed using doxorubicin. A doxorubicin treatment procedure was established using 0.5μM doxorubicin for 2hrs followed by a recovery for 24hrs. BrdU was added for the final 4 hrs and the cells analysed by flow cytometry to assess their cell cycle profile. Cells with functional 14-3-3σ pathways should arrest while those with silenced 14-3-3σ should not. Therefore, as controls we treated MCF7 and MDA-MB-435 cell lines. MCF7 cells have unmethylated and transcribed 14-3-3σ whereas MDA-MB-435 cells have methylated and silenced 14-3-3σ. Interestingly, both MCF7 and MDA-MB-435 cells are arrested upon treatment with doxorubicin, although the nature of the arrest is different for each cell type. MCF7 cells with functional 14-3-3σ are arrested in both G1 and G2 (Fig. 4). MDA-MB-435 cells are arrested in G2 after doxorubicin treatment, with a reduction in the number of cells in G1 and S-phase. Since 14-3-3σ is methylated and silenced in MDA-MB-435 cells, the G2 arrest is presumably independent of 14-3-3σ function.

A

	DNA content			
	%G1	%S	%G2	%>4N
MCF7 sham	41.1	49.0	4.7	2.7
MCF7 dox	60.5	18.0	12.8	4.3
	DNA content			
	%G1	%S	%G2	%>4N
435 sham	24.2	66.2	2.7	4.7
435 dox	10.0	18.5	57.3	8.8

B

	DNA Content			
	%G1	%S	%G2	%>4N
HMEC sham	75.1	13.5	9.29	0.52
HMEC DOX	75.9	4.41	17.9	7.73E-03
HMEC sham	59.2	19.4	15.4	3.1
HMEC DOX	58.9	9.28	22.9	2.61
	DNA Content			
	%G1	%S	%G2	%>4N
vHMEC sham	59.7	15.8	17.3	3.81
vHMEC DOX	56.2	4.73	33.1	2.27
vHMEC sham	66.1	11.6	18.3	2.53
vHMEC DOX	48.9	6	39.5	3.79

Figure 4: Doxorubicin induces a cell cycle arrest. (A) MCF7 and MDA-MB-435 cells or (B) variant HMEC were grown on plastic and doxorubicin (0.5μM final, DOX; or sham treatment) was added for 2 hrs and the cells allowed to recover for 24hrs prior to harvesting. BrdU was added for the final 4 hrs. The cells were then collected and stained for BrdU and PI, and cell cycle fractions determined.

HMEC and vHMEC were then treated with doxorubicin. Interestingly, both untreated HMEC and vHMEC had a reduced S-phase fraction as compared to MCF7 and MDA-MB-435 cells. Upon treatment, HMEC showed a decreased S-phase, no change in

G1 and an increase in G2, whereas vHMEC showed a decreased S-phase, a slight decrease in G1 and a similar increase in G2 to that seen in HMEC (~2 fold increase). In both cases, the numbers of cells escaping the G2 arrest would be expected to give a methylation product by MSP, which was not the case (Fig 1). Therefore, doxorubicin treatment was not a suitable method to induce a tight G2 arrest in vHMEC.

Specific aim (a) To determine the frequency of 14-3-3σ promoter methylation in human mammary epithelial cells that are grown in tissue culture in 3 dimension (in matrigel)

In order to determine the frequency of 14-3-3σ promoter methylation in human mammary epithelial cells grown in 3 dimensions, we cultured both HMEC and vHMEC (Romanov et al. 2001; Bannister et al. 1998) in a reconstituted basement membrane substrate (rBM; matrigel, BD Biosciences). In matrigel both HMEC and vHMEC form 3D polarized spheres similar to human mammary acini. These matrigel structures were grown in MEGM medium and 1-2x10^6 cells harvested from the matrigel. DNA was extracted with a DNA extraction kit (Promega) and 1-2μg of genomic DNA was bisulfite modified as described (Herman et al 1996). Conversion was optimized in our hands such that an incubation at 55 C for 5 hours was used. We then carried out methylation-specific PCR on bisulfite treated DNA from both HMEC and vHMEC. Primers were designed as described (Ferguson et al 2000) to amplify either methylated or unmethylated alleles of the 14-3-3σ promoter. The conditions that gave specificity for methylated and unmethylated alleles were 95 C for 5 minutes followed by 31 cycles of 95 C for 30 seconds, 56 C for 30 seconds, 72 C for 30 seconds, followed by an extension cycle of 72 C for 4 minutes.

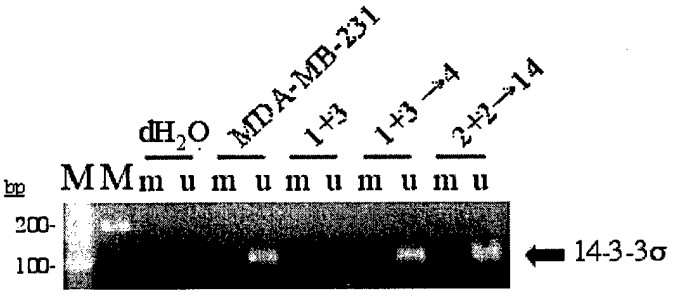

Fig.5: Methylation-specific PCR at the 14-3-3σ promoter in cells grown in 3-D. (A) Assessment of methylated (m) and unmethylated (u) alleles from MDA-MB-231, HMEC (1+3), vHMEC early (1+3→4) and late (2+2→14) passage of RM16. Marker (M) lanes are indicated.

As expected, bisulfite modified DNA from MDA-MB-231 cells only gave products with unmethylated primer sets. Bisulfite modified HMEC DNA only gave products with unmethylated primer sets (Fig. 5). Bisulfite modified DNA from vHMEC also only gave products with unmethylated primer sets. Experiments are ongoing to determine the detectable frequency of methylated alleles in this assay. Thus, we found that vHMEC grown in 3 dimensions (in matrigel) have unmethylated alleles of 14-3-3σ as detected by methylation-specific PCR.

Specific aim (b, c)

To determine the frequency of this same event after the cells have been grown in association with fibroblasts from normal mammary tissue or adenocarcinoma.

Co-culture of fibroblasts and epithelial cells grown on plastic or in matrigel were successfully carried out. On plastic, fibroblasts provide a confluent lawn of cells upon which the epithelial cells proliferate. Cell-cell interactions which modulate epithelial cell proliferation or death can easily be measured. In matrigel, the epithelial cells form mammospheres and ductal structures that are surrounded by adjacent fibroblasts. To assess the frequency of 14-3-3σ hypermethylation in epithelial cells techniques developed in specific aim (a) were used. However, since we show that mammary fibroblasts have a proportion of methylated alleles (RM21 fibroblasts), this confounds analysis of the methylation status of epithelial alleles by the presence of contaminating fibroblast alleles. Experiments are ongoing to successfully purify epithelial cell populations from co-cultures that could be then be assessed using the methylation-specific PCR assay. Using cell-labeling techniques we will attempt to recover populations of epithelial cells that would have a low enough frequency of contamination with fibroblasts to be used in methylation-specific PCR.

KEY RESEARCH ACCOMPLISHMENTS

The finding that 14-3-3σ promoter methylation is not a frequent event in vHMEC, although it is frequently methylated in both early and late stage breast cancer.

MSP analysis of the 14-3-3σ locus can be used to distinguish mammary epithelial cells from mammary fibroblasts

REPORTABLE OUTCOMES

Presentations/ abstracts:

AACR Special Conference
Chromatin, Chromosomes, and Cancer Epigenetics
November 10-14, 2004
Hilton Waikoloa Village
Waikoloa, Hawaii

Invited speaker presentation:
Epigenetic Contributions to Early Breast Cancer
Thea D. Tlsty, University of California, San Francisco, CA

Poster presentation:
"Investigating promoter hypermethylation in human mammary epithelial cells"
Paul A. Reynolds, Mahvash Sigaroudinia, and Thea D. Tlsty

People who worked on grant specifc aims:

Dr. Paul Reynolds
Visiting Postdoctoral Scholar

CONCLUSIONS

We have successfully established a methylation-specific PCR assay to distinguish methylated from unmethylated alleles of the 14-3-3σ promoter. We found that 14-3-3σ is unmethylated in normal HMEC. We further determined that vHMEC postulated to be a very early step in breast carcinogenesis also lacks appreciable hypermethylation of 14-3-3σ promoter sequences. Interestingly, 14-3-3σ is methylated in mammary fibroblasts. To address whether mammary stroma affects the frequency of methylation in mammary epithelial cells using our established methylation-specific PCR assay, we must optimize separation techniques to separate fibroblasts (with methylated 14-3-3σ sequences) from rare epithelial cells that may have hypermethylated the same sequences.

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