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Table of Contents

Cover..... 1

SF 298..... 2

Table of Contents..... 3

Introduction..... 4

Body..... 4

Key Research Accomplishments..... 9

Reportable Outcomes..... 9

Conclusions..... 10

References..... 12

INTRODUCTION

Our hypothesis is that the IGF2 gene is methylated in normal breast stromal cells, and that a decrease in methylation results in increase in stromal IGF2 expression in the breast malignancy, enhancing tumor growth. This work analyzes the biochemical mechanisms that alter methylation of the IGF2 promoter in benign stromal cells adjacent to the malignant epithelial cells in the breast tumor. The studies of this project were conducted in two systems: T61 breast cancer system, an ER+, PR+, IGF2 expressing breast cancer xenograft passaged in nude mice, and matched fibroblast sets from breast cancer patients. These studies could provide critical insight into stromal regulation of breast cancer and provide a new target for therapy.

BODY: Control Of Expression Of Insulin-Like Growth Factor 2 In Stromal Cells Of Breast Cancer

Technical Objective A: Determine which promoters drive IGF-II expression in stromal cells in normal and malignant breast tissue. (Months 1-18)

1. Determine methylation status of individual IGF-II promoters. (Months 1-12)

Based on the results of the promoter usage **(see Objective A.2)** in these breast cancer systems, methylation analysis was done by Southern blot analysis using a radioactive probe against the P4 promoter. Results of analysis of *HpaII* and *MspI* digests of the T61 tumor tissue treated with estradiol indicate that there is a higher migratory species at later timepoints compared to the T0 timepoint **(Figure1)**. This fragment may in fact be a result of methylation which would inhibit digestion by the *HpaII* endonuclease of the last four restriction sites covered by this probe. This would result in the evident 779bp fragment. The surprising consequence is the presence of the same fragment in the *MspI* lane. *MspI* endnuclease should digest each of its restriction sites, regardless of the presence of methyl groups. However, the restriction sites under consideration are close to each other, between 50 and 100 bp apart. The proximity of the methyl moities in this region, possibly due to secondary structure of the genomic DNA, may result in a steric hindrance that could hamper *MspI* as well as methylation sensitive *HpaII*.

Initial analysis of the methylation status of the P4 promoter region in this matched fibroblast set demonstrates similarities to the results seen in the T61 samples. **(Figure 2)** Two faint bands were observed in the *HpaII* lane of the G50 tumor fibroblast line at approximately

330bp(153+57+110) and 370bp(153+57+110+53), indicating possible methylation at two and three restriction sites within the P4 promoter region. Interestingly, this may represent a loss of methylation at the sites between fragments 153 and 57 as well as 57 and 110. Moreover, there are two higher migratory species in the *HpaII* and *MspI* digest lanes of the G50 PF line. The sizes of this second set of bands, 722bp(110+53+106+453) and 779bp(57+110+53+106+453), imply that the last two restriction sites covered by the P4 probe are methylated. Like the later timepoints of the T61 tumor treated with estradiol, both *HpaII* and *MspI* show the slower migratory moieties. There may be a similar explanation for this phenomenon as in the T61 system. That is, DNA coiling may bring these last four restriction sites within the vicinity of each other and result in impedance of *MspI* as well as *HpaII* action. This study will require further investigation to give conclusive results.

2. Determine promoter usage in breast tumor stromal cells, normal stromal cells and breast epithelial cells. (Months 6-18)

RNase protection assays were done to determine the expression levels of the four different transcripts of the IGF2 gene. The four transcripts each have a unique 5' region and share exons 7, 8, and 9. Riboprobes were generated by PCR amplification of distinct regions of the IGF2 gene. These include: (1) P1: 222bp fragment: unique region of exon 3 of the IGF2 gene; (2) P2: 469 bp fragment: 200bp of unique region of exon 4, and 269 bp including exon 7 and 8; (3) P3: 333 bp fragment: unique region of exon 5; and (4) P4: 465 bp fragment: 20bp unique region of exon 6, and 445 bp fragment including exon 7,8, and 9. The positive controls for each are as follows: P1: total RNA from adult liver (Clontech); P2: total RNA from perinatal liver (Clontech); P3 and P4: total RNA from RD rhabdomyosarcoma cells.

Total RNA samples from T61 tumor tissue excised at 0, 3, 6, 24, and 96 hours after administration of estradiol were assayed for IGF2 transcript mRNA expression. There was no detectable P1 transcript expression in any of the timepoints analyzed. On the other hand, the bottom gel shows that the P2 transcript levels seem constant through early treatment timepoints. P2 expression falls to barely detectable levels between 48 and 96 hours after estradiol treatment. **(Figure 3)**

The P3 transcript gel shows very faint bands at each of the timepoints that appear to follow the dynamic of IGF2 mRNA expression. However, by comparing these bands to the 36B4

mRNA expression, we observed that the P3 transcript mRNA expression is not significant. The P4 riboprobe gave a surprising result. The probe itself includes only 20bp of unique P4 transcript; the other 445bp are exons 7, 8 and 9 which all the IGF2 promoter transcripts have in common. Therefore, our initial consideration of the result from the P4 riboprobe hybridization was that this represents a subtractive element. That is, the bands on the P4 gel would represent common IGF2 transcript from among the four promoters. Using the 36B4 riboprobe as the normalizing factor in the assay, a distinct doublet of bands was observed at each of the timepoints that were assayed. **(Figure 4)** The bottom band, which migrates to 445bp, represents the common IGF2 transcript and probably includes the P2 and P3 transcripts. However, the top band, which migrates at 465bp, is unique P4 transcript. P4 mRNA levels also follow the dynamic of IGF2 expression previously demonstrated. P4 expression appears to decrease at later timepoints, between 24 and 96 hours after drug treatment.

Total RNA from the matched fibroblast set from patient G50 was also analyzed. As in the case of the T61 samples, the tumor fibroblast sample demonstrated predominant expression of the P2 and P4 transcripts. There was no detectable expression of P1 or P3 transcript in either fibroblast line. The peripheral fibroblast sample (G50PF) did not show expression of any of the IGF2 transcripts. This negative result shows that the apparent P2 and P4 transcript expression was specific to IGF2 expressing tissue and cells. **(Figure 5)**

Technical Objective B: Determine the correlation between DNA (cytosine-5) methyltransferase (DNMT) and IGF-II expression in breast tumor stromal cells, normal breast stromal cells and malignant breast epithelial cells. (Months 1-24)

1.Determine DNMT mRNA levels by RNAse protection assay. (Months 1-12)

We have previously demonstrated that there is modulation of IGF2 expression in two breast cancer systems: T61 tumor xenograft and matched fibroblasts sets from breast cancer patients. Here, we have attempted to show that there is a correlation in DNMT1 mRNA expression and activity and IGF2 expression. As indicated, there is a very strong possibility that IGF2 and DNMT1 are reciprocally expressed in breast cancer. We found that in the T61 xenograft, in which IGF2 expression is reduced, there is an increase DNMT1 mRNA expression over time to twofold at 96 hrs after administration of estradiol,**(Figure 6)**. In the matched fibroblast set from breast cancer patient G50, there was a decrease in DNMT1 mRNA expression

in the tumor fibroblast line to 40% of the level observed in the peripheral fibroblast line. This coincides with the evident expression of IGF2 mRNA in the tumor fibroblast lines which is undetectable in the peripheral fibroblasts analyzed from breast cancer patients G54 and G50. **(Figure 7)**

2.Exposure of stromal cells to epithelial cell-conditioned media and determination of DNMT expression. (Months 12-24)

These experiments will be conducted in the near future.

3.Determine DNMT enzymatic activity in tumor fibroblasts and peripheral fibroblasts. (Months 6-18)

In the samples from the T61 tumor tissue treated with estradiol, there was a threefold increase in DNMT1 activity as early as three hours after the drug treatment. **(Figure 8)**. The difference in DNMT1 activity between the peripheral and tumor fibroblasts was very surprising. There was as much as one log order higher activity in the peripheral fibroblasts as compared to the tumor fibroblasts. **(Figure 9)**

Technical Objective C: Modulate IGF-II expression by chemical and molecular manipulation of DNMT in these cells. (Months 12-24)

1.Treatment of breast epithelial and stromal cells with agents that modulate DNMT expression (5-Azacytidine) and determination of IGF-II mRNA levels. (Months 12-24)

We then addressed regulation of IGF2 expression by modulation of DNMT1 function. Based on the previous data, we hypothesized that inhibiting DNMT1 function would cause an increase in IGF2 mRNA expression. Our experiments involved treating peripheral breast fibroblasts with 5-Azacytidine. We expected that the 5-Azacytidine treatment of the peripheral fibroblasts would bring about an increase in IGF2 mRNA expression, mimicking the possible process occurring during breast tissue transformation. Our control experiments were done with HepG2, T47D and MDA-468 immortalized cell lines. Interestingly, the doses we established as capable of decreasing DNMT1 activity altered IGF2 expression based on the cell line. **(Figure 10)** There was a slight increase (5-10%) in IGF2 expression in the HepG2 cell line and in the T47D cells. However, the MDA-468 cells did not appear to be affected by the doses of 5-

Azacytidine (as high as 250nM) administered, although they were within proximity of the IC50 dose(500nM). **(Figure 11)** This result may indicate that DNA methylation may not be the sole regulatory mechanism of IGF2 gene expression. Other components, including histone deacetylation as well as transactivation by factors such as Egr-1, are probably involved in the regulation of IGF2 expression in these immortalized cell lines.

These results reflected what we observed in the peripheral fibroblast cell lines, G54PF and G60 PF. A dose of 0.75uM 5-Azacytidine was administered to the G54 PF cells. However, there was no apparent increase in IGF2 mRNA expression as expected. In fact, DNMT1 activity analysis revealed that the 0.75uM dose caused an increase in DNMT1 activity as compared to the untreated cells. This is an indication of the toxicity mechanism. Treatment with a cytotoxic drug, which affects enzyme function, can cause the target to increase its function in an effort to overcome the effect of the drug. This phenomenon was observed in MDA-468 cells treated with a low dose (62.5 nM) 5-Azacytidine. **(Figure 12)** A higher dose of drug usually eliminates this phenomenon. However, treatment of G60 PF cells with 1uM 5-AzaC did not result in an increase in IGF2 mRNA expression, indicating that other mechanisms may be necessary to alleviate the epigenetic control of IGF2 expression. **(Figure 13)**

2. Treatment of breast epithelial and stromal cells with agents that modulate Ras activation pathway and determine effects on IGF-II mRNA expression. (Months 12-24)

These experiments will be conducted in the near future.

KEY RESEARCH ACCOMPLISHMENTS:

- Demonstrated predominant promoters of IGF2 gene that drive expression in breast cancer
- Demonstrated DNMT1 mRNA expression in two breast cancer models
- Demonstrated DNMT1 activity in two breast cancer models

REPORTABLE OUTCOMES:

1. Cullen KJ, Kaup SS, and Rasmussen,AA. Interactions between Stroma and Epithelium in Breast Cancer: Implications for Tumorigenesis, Grwth, and Progression. In Mann A (ed) Endocrinology of the Breast, Kluwer Publishers, 1998

2. Sahana S Kaup, Nils Brunner, Audrey A Rasmussen, Kevin J Cullen. 2000. Control Of Expression Of Insulin-Like Growth Factor II In Breast Cancer. Abstract in 2000 Proceedings of the American Association of Cancer Research.

3. Sahana S Kaup, Nils Brunner, Audrey A Rasmussen, Kevin J Cullen. 2001. Regulation Of Expression Of Insulin-Like Growth Factor II In Breast Cancer. Abstract in 2001 Proceedings of the American Association of Cancer Research.

CONCLUSIONS:

We have demonstrated the following:

- IGF2 expression is driven by the P2 and P4 promoters in the two breast cancer models analyzed.
- There is an increase in methylation at the 5' end of the IGF2 promoter P4 region studied in the T61 tumor system treated with estradiol.
- There is a change in methylation in same region of the IGF2 promoter P4 in the tumor fibroblasts compared to the peripheral fibroblasts from breast cancer patient G50.
- In the estradiol-treated T61 tumor model, DNMT1 mRNA expression increases to twofold of original T0 levels and IGF2 mRNA expression decreases to 30 % of T0 levels.
- In the matched fibroblast set from breast cancer patient G50, there was a decrease in DNMT1 mRNA expression in the tumor fibroblast line to 40% of the level observed in the peripheral fibroblast line. This coincides with the evident expression of IGF2 mRNA in the tumor fibroblast lines which is undetectable in the peripheral fibroblasts analyzed from breast cancer patients G54 and G50.
- In the estradiol-treated T61 tumor model, DNMT1 activity increases to threefold of T0 levels at 3 hours after administration of estradiol and remains at this level through later timepoints.
- There was as much as one log order higher activity in the peripheral fibroblasts as compared to the tumor fibroblasts.
- The treatment doses of 0.75 uM and 1.0 uM 5-Azacytidine, an inhibitor of DNMT1, was not sufficient to induce IGF2 expression in the fibroblast cell lines analyzed. It is possible that other regulatory factors, such as histone deacetylation, are involved in regulation of IGF2 gene expression in breast cancer.

Our studies indicate that DNMT1 plays a significant role in IGF2 expression in breast cancer, as has been shown in other mesenchymal systems that express IGF2. This project has provided a novel introduction into the mechanisms that regulated IGF2 expression in breast stromal cells. These results imply that genomic imprinting is a key form of regulation of IGF2 gene expression in breast stromal cells and will help guide future research in this subject. Future directions of this project may include that impact of the other component of genomic imprinting, histone deacetylation, on IGF2 gene expression in this system. Also, understanding the influence of genomic imprinting on IGF2 gene expression can aid in the treatment of breast cancer by including drugs, such as 5-Azacytidine, that deter this form of gene regulation.

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2. Brunner, N., Yee, D., Kern, F. G., Spang-Thomsen, M., Lippman, M. E., and Cullen, K. J. Effect of endocrine therapy on growth of T61 human breast cancer xenografts is directly correlated to a specific down-regulation of insulin-like growth factor II (IGF-II). Eur.J.Cancer, *29A*: 562-569, 1993.

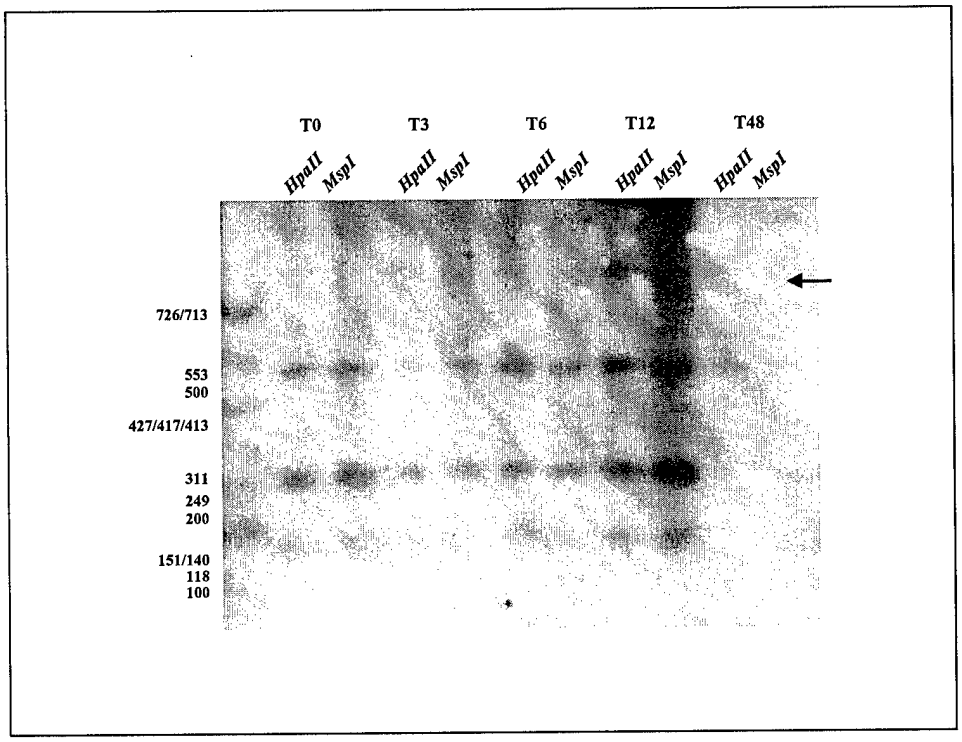

Figure 1:Southern Blot Analysis of P4 region of human IGF-II gene in samples of T61 tumor tissue treated with estradiol. This blot confirms the results of Figure 12. It represents the result of hybridization with the P^{32}-radiolabelled P4 probe of electrophoresed genomic DNA samples from each timepoint T0, T3, T6, T12, andT48 after *HpaII* and *MspI* restriction digest. There is a band at around 779bp(←) in later timepoints compared to the T0 timepoint. Given the predicted fragment sizes if each site was used, this 779bp species probably includes that last four restriction sites covered by the P4 probe.

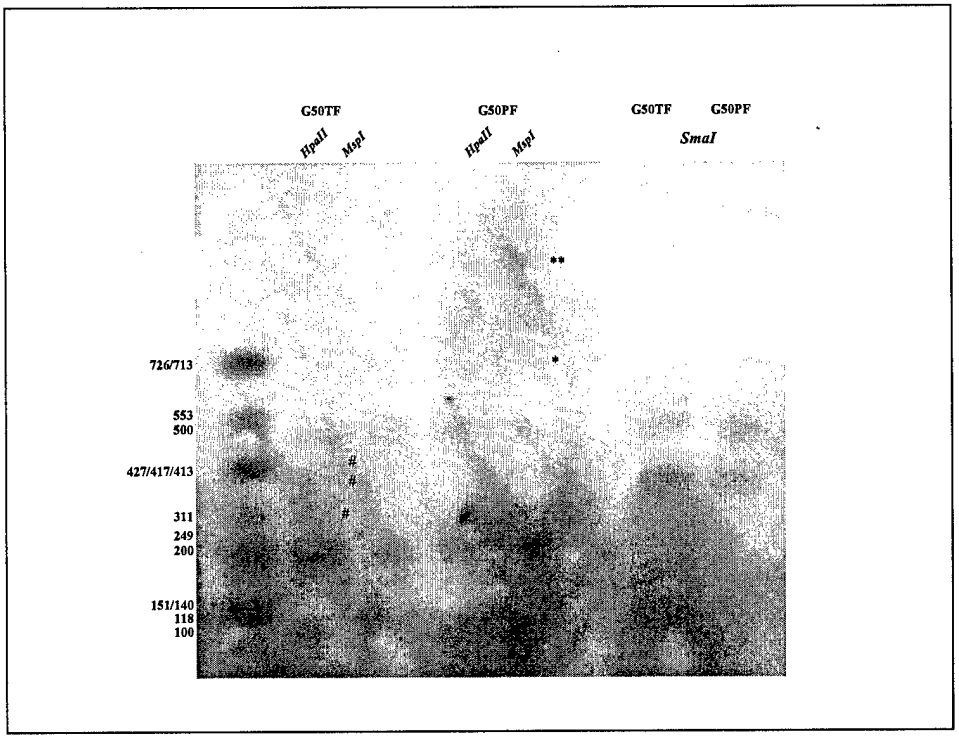

Figure 2:Southern Blot Analysis of P4 region of human IGF-II gene in samples of matched fibroblast set from breast cancer patient G50. This blot represents the result of hybridization with the P^{32}-radiolabelled P4 probe of electrophoresed genomic DNA samples from tumor **(G50TF)** and peripheral**(G50PF)** fibroblasts after restriction digest with indicated endonucleases.The *HpaII* lane of G50TF shows a different banding pattern compared to the *MspI* lane of the same line, indicating methylation of two **(330bp: #)** and three **(370 bp:##)**restriction sites within the region detected by the P4 probe. There are two bands at **722(*)** and **779bp (**)** in *HpaII* and *MspI* lanes of G50PF line, suggesting the last two restriction sites of the region covered by the P4 probe are methylated.

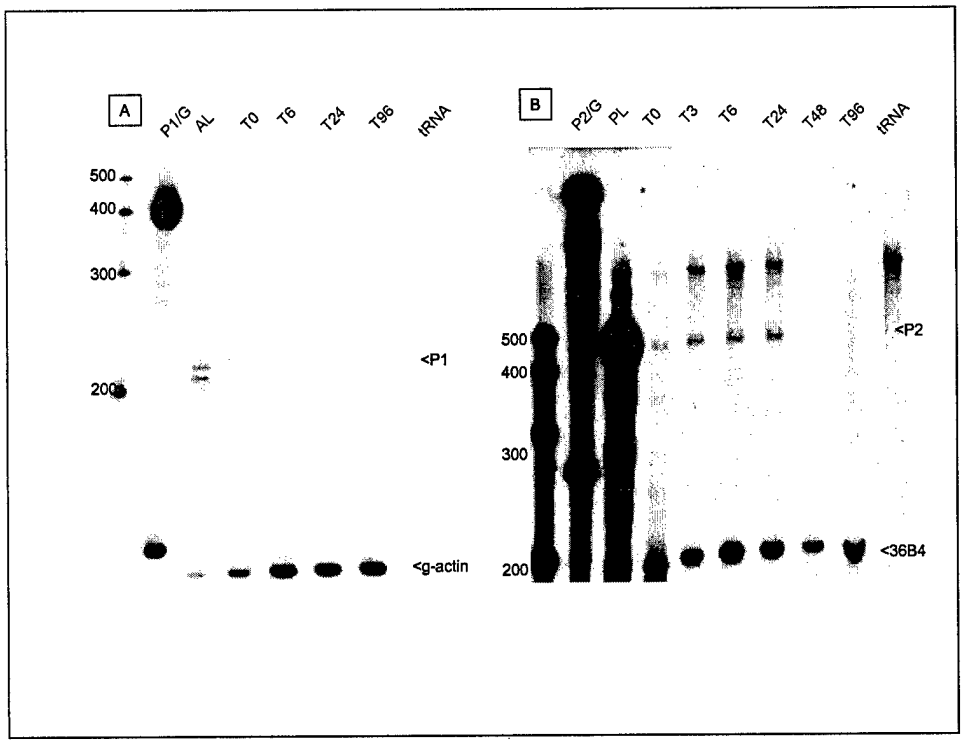

Figure 3: Promoter Usage of P1, and P2 of the IGF2 gene in T61 tumor treated with Estradiol(E2). These gels represent the analyses of promoter expression from promoter P1, P2 and P3 of the IGF2 gene. **Gel A** shows P1 expression using 20 ug total RNA from adult liver (Clontech) as a positive control. There is no expression of this transcript in this breast cancer system. **Gel B** demonstrates significant P2 expression in this breast cancer system. The positive control for P2 expression was 20 ug total RNA from perinatal liver (Clontech)

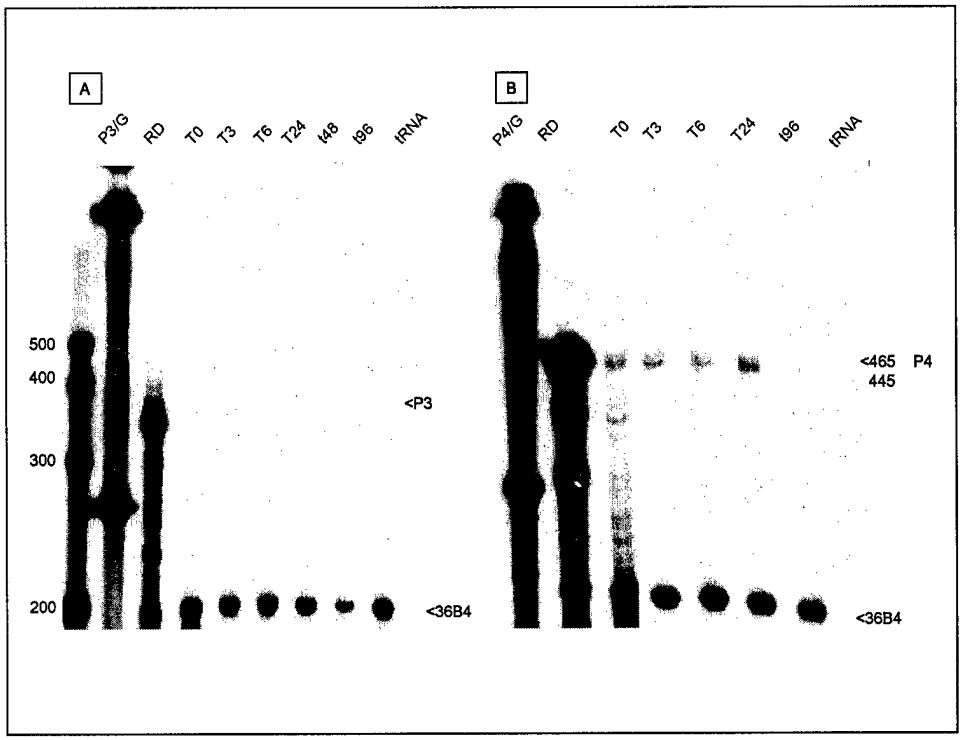

Figure 4: Promoter Usage of P3 and P4 of the IGF2 gene in T61 tumor treated with Estradiol(E2). These gels represent the analyses of promoter expression from promoter 4 of the IGF2 gene. **Gel A** shows P3 expression using 20ug total RNA from RD rhabdomyosarcoma cells as a positive control. There are low levels of P3 transcript expression which are insignificant when normalized to 36B4 mRNA expression. **Gel B** for which P4 transcript mRNA expression was normalized against 36B4 mRNA expression, demonstrates that the P4 riboprobe detects two species: the top band (465bp) indicates unique P4 transcript. The bottom band (445bp) protects the region including exons 7,8 and a portion of 9 of the IGF2 gene and therefore detects common IGF2 transcript from the P2 and/or P3 promoters.

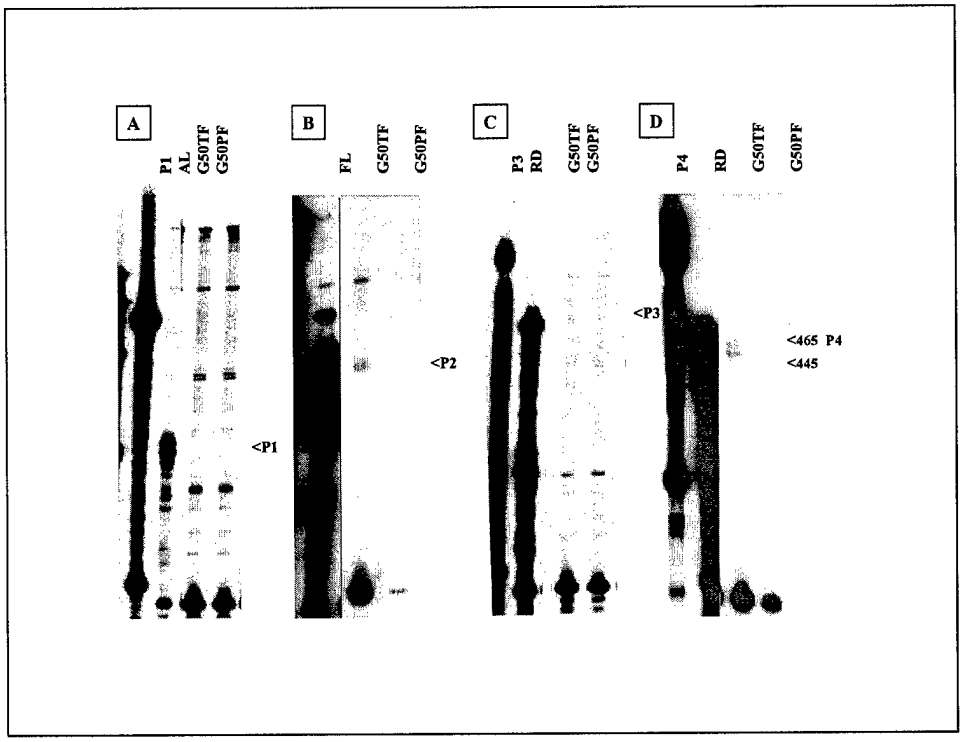

Figure 5: Promoter Usage in the Matched Fibroblast Set From Breast Cancer Patient G50. These gels represent analyses of promoter usage in tumor and peripheral (normal) fibroblasts from breast cancer G50. **Gel A** shows there is no P1 mRNA expression in either fibroblast line. **Gel B** indicates that there is P2 mRNA expression in the tumor fibroblast line but not the peripheral fibroblast line. **Gel C** shows no P3 expression in either cell line. **Gel D** clearly shows that the tumor fibroblasts express P4 transcript (the doublet of 465 and 445 bp) but the peripheral fibroblasts do not.

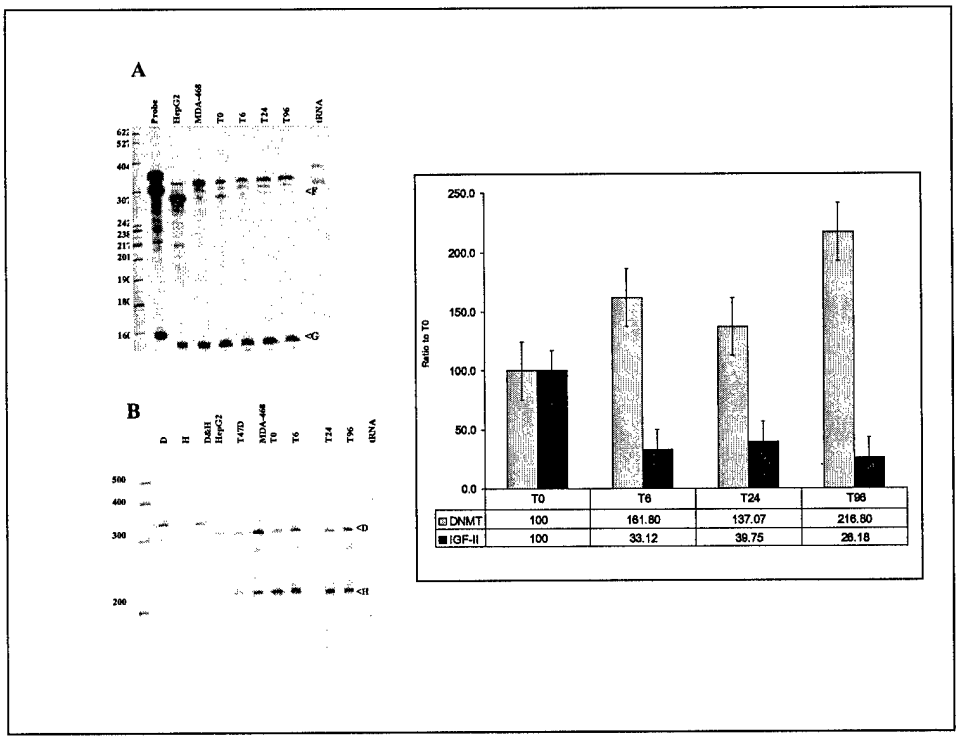

Figure 6: IGF-II and DNMT1 mRNA Expression in T61 Tumor Tissue Treated with Estradiol. Gel A represent IGF-II mRNA(**F**) and γ-actin mRNA(**G**) expression in archived samples of T61 tumor tissue treated wiith estradiol. As previously described (Brunner et al)(**graph 18a**), there is a decrease in IGF-II mRNA at 6 hours after drug treatment. **Gel B** represents the DNMT1 mRNA (**D**) and Histone H4 (**H**) expression in these samples. They are quantified in **graph a** and indicate that DNMT1 mRNA expression increases gradually after administration of estradiol, reaching a twofold level of expression at 96 hours after drug treatment. **Graph b** shows that assay of recently treated samples of T61 tumor tissue demonstrate a similar trend in IGF-II and DNMT1 mRNA expression.

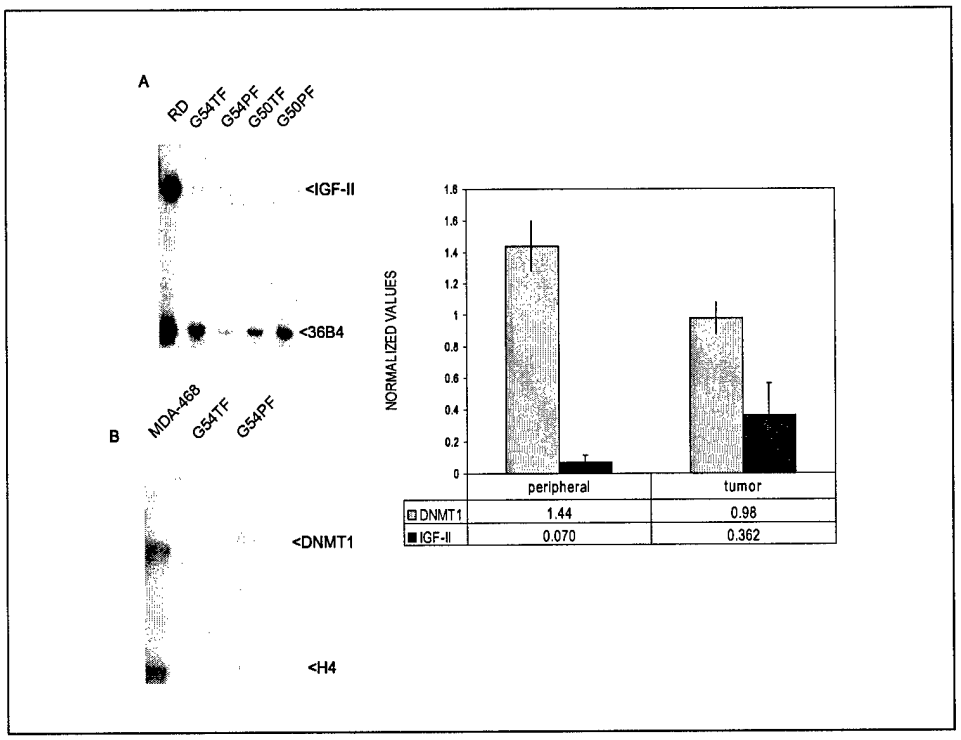

Figure 7: IGF-II and DNMT1 mRNA Expression in Tumor and Peripheral Fibroblasts from Breast Cancer Patients G54 and G50.Gel A represents IGF-II and 36B4 mRNA expression and **Gel B** represents analysis of DNMT1and Histone H4 mRNA expression in tumor fibroblast (G54TF and G50TF) and peripheral (G54PFand G50PF) fibroblast lines from breast cancer patient G54 and G50.The adjacent graph indicates the reciprocal expression between IGF-II and DNMT1 mRNA demonstrated in the first set of RNA samples from breast fibroblasts analyzed.

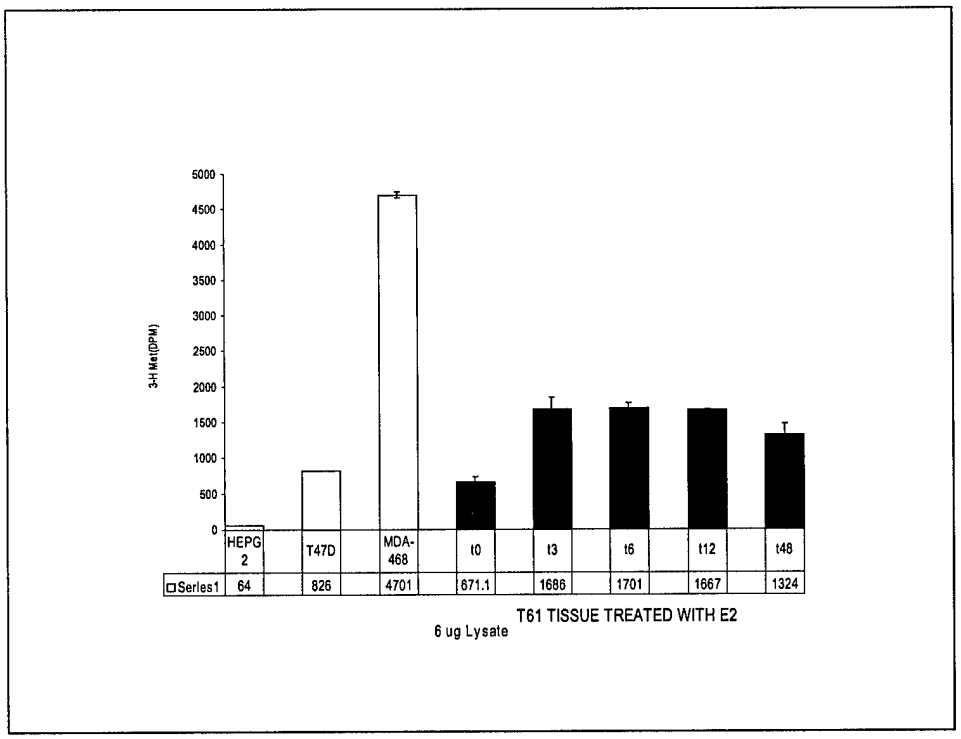

Figure 8: DNMT1 Activity in Immortalized Cell Lines and Crude Lysates from T61 Tumor Tissue Treated with Estradiol. The graph bars in yellow indicate the levels of DNMT1 activity measured in HepG2 hepatocellular carcinoma cells, T47D and MDA-468 breast cancer cells. These activity levels were commensurate with the DNMT1 mRNA levels assayed. The maroon graph bars demonstrate the DNMT1 activity levels in 6 ug crude lysate from timepoints t0, t3, t6, t12, and t48 of T61 tumor tissue treated with estradiol. There is a threefold increase in DNMT1 activity, as compared to the t0 timepoint , as early as three hours after drug treatment and this level is constant through later timepoints.

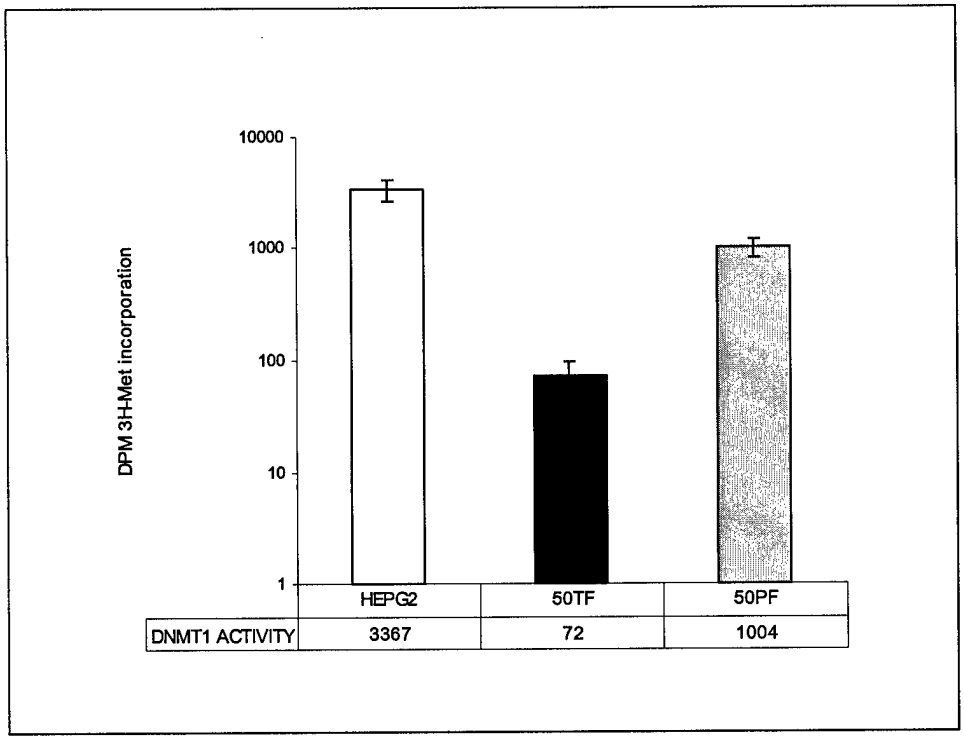

Figure 9: DNMT1 Activity in Immortalized Cell Lines and Crude Lysates from HS27 human foreskin fibroblasts and peripheral fibroblasts from breast cancer patients G54 and G50. This graph is on a log scale. The graph bars in yellow indicate the levels of DNMT1 activity measured in HepG2 hepatocellular carcinoma cells, T47D and MDA-468 breast cancer cells. These activity levels were commensurate with the DNMT1 mRNA levels assayed. The green graph bar demonstrates the DNMT1 activity level in 6 ug crude lysate from HS27 human foreskin fibroblasts, an IGF-II expressing fibroblast line. The lavender graph bars demonstrate the DNMT1 activity level in 6 ug crude lysate from peripheral fibroblasts from breast cancer patients G54 and G50. These lines do not express IGF-II. DNMT1 activity is twofold higher in the peripheral fibroblasts compared to the HS27 fibroblasts.

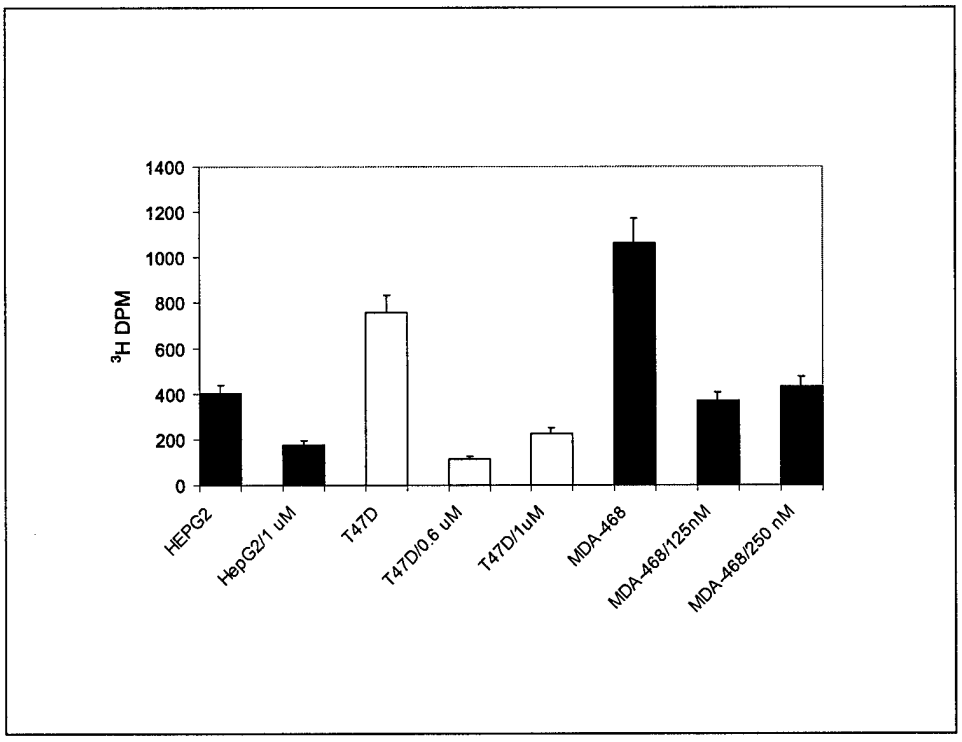

Figure 10: DNMT1 Activity in Immortalized Cell Lines Treated with 5-Azacytidine. HepG2 , T47D and MDA-468 cells were treated with 5-Azacytidine at doses below IC50 dose. The graph indicates that these doses were sufficient to decrease DNMT1 activity in these lines.

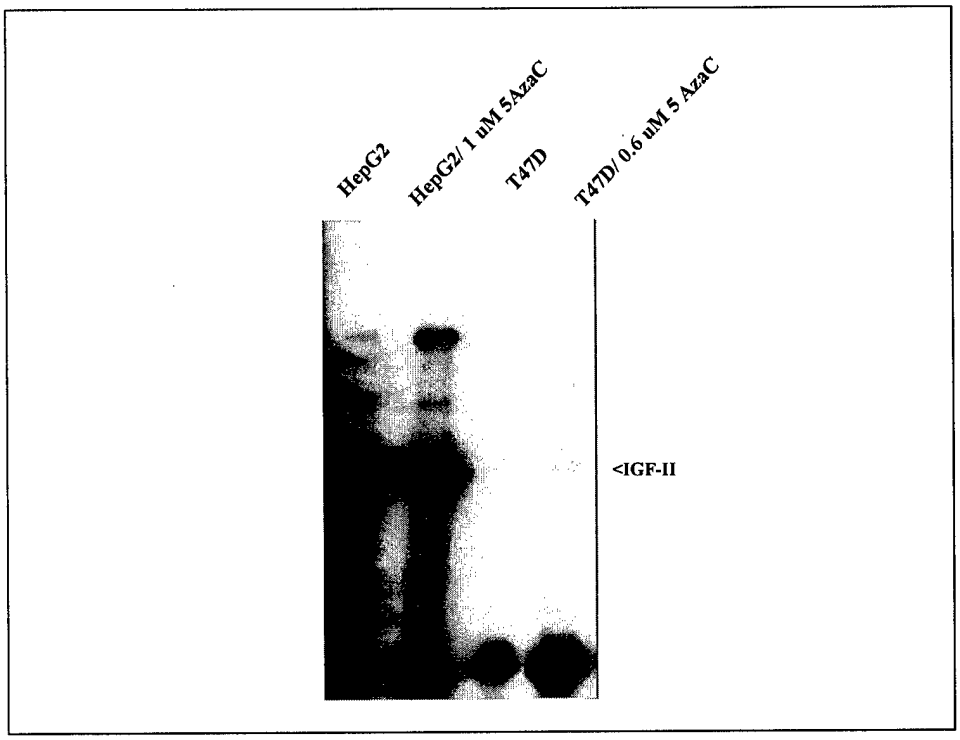

Figure 11: IGF-II mRNA Expression in Immortalized Cell Lines Treated with 5-Azacytidine. The gel represents RNase protection assay of 20ug total RNA from untreated and treated HepG2 and T47D cells. It demonstrate a slight increase in IGF-II mRNA expression when they were treated with 5-Azacytidine at doses below IC50 dose. Quantification was done by densitometry. Normalized values indicate that there is a 5-10% increase in the cell lines treated with 5-Azacytidine. There was no detectable effect on the MDA-468 cells.

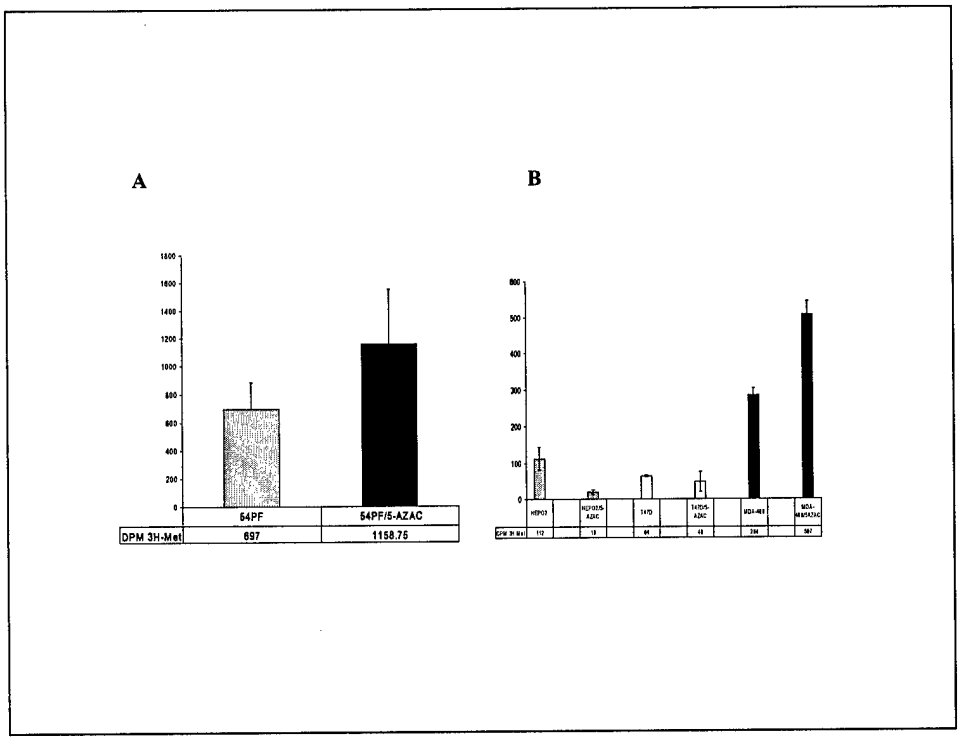

Figure 12: Peripheral Fibroblasts from Patient G54(G54PF) Treated with 5-Azacytidine. Graph A demonstrates the DNMT1 activity in untreated G54 PF cells and those treated with 0.75 uM 5-Azacytidine. There is an increase in DNMT1 activity with this dose, indicating a toxicity effect. **Graph B** shows that a similar effect was observed when a very low dose of 5-Azacytidine was administered to MDA-468 cells. Based on the results of this initial DNMT1 activity assay, 5-Azacytidine doses were doubled. The results of application of this subsequent dose is shown in **figure 26.**

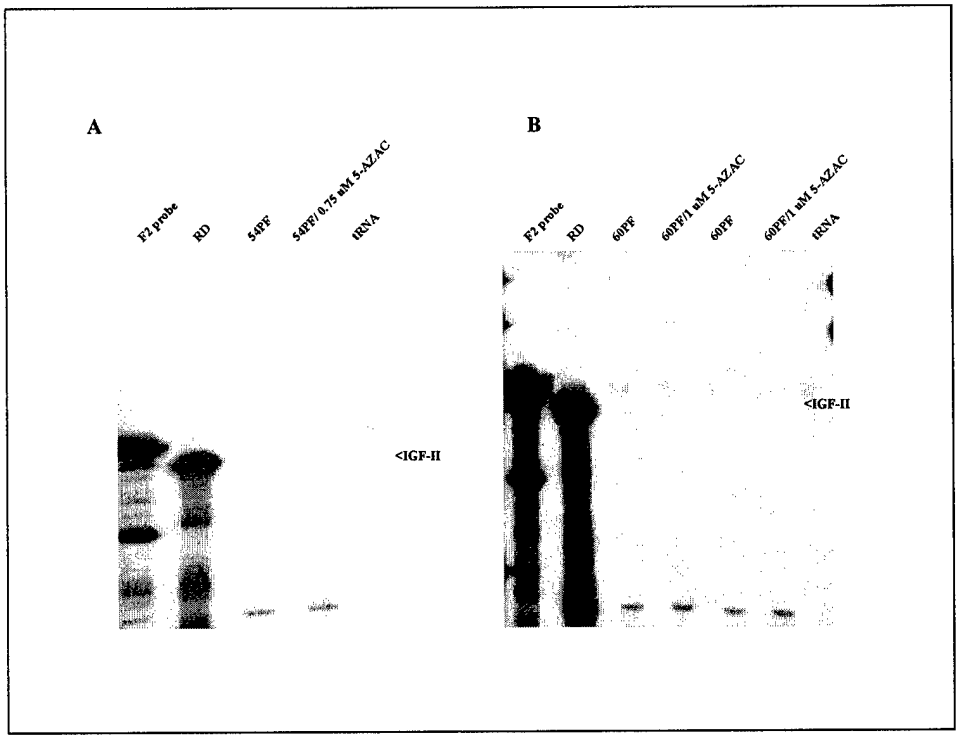

Figure 13: Peripheral Fibroblasts from Breast Cancer Patients G54 and G60 Treated with 5-Azacytidine. Gel A demonstrates that there is no increase in expression in G54 PF cells treated with 0.75 uM 5-Azacytidine. Increasing the dose to 1 uM (**Gel B**) does not cause an increase in IGF-II expression in another peripheral fibroblast line (**G60 PF**), indicating that other factors may be involved in the regualtion of IGF-II expression in these cells.