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SP1
DOCK4 IS FREQUENTLY EXTINGUISHED IN SPORADIC BREAST CANCER
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Loss of heterozygosity at 7q31 is among the most frequent genetic abnormalities associated with sporadic breast cancer. The tumor suppressor gene, dedicator of cytokinesis 4 (DOCK4), is involved in the induction of adherens junctions resides at this locus. The aim of this study was to analyze the expression of this gene in a panel of normal (n= 18), and sporadic breast cancer tumor specimens (n=34), and compare its expression with parameters of grade and stage of the disease. DOCK4 mRNA was detected in 15/18 (83%) of normal specimens and 16/34 (47%) of the breast tumor specimens (c2= 6.43, $p<0.025$). No correlation was observed between the stage of the tumor and expression of the DOCK4 gene ($P <0.3$). For the first time, these findings implicate that DOCK4 is frequently extinguished in the course of tumorigenesis of breast cancer. This may explain a mechanism of evasion from contact inhibition in this type of cancer.

SP2
IMAGING OF TUMOR-TARGETING QUANTUM DOT IN BREAST CANCER OF LIVING MICE
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Recent anti-cancer therapeutics has been based on an active tumor targeting by conjugating antibodies against tumor-associated antigens such as HER2, for increasing therapeutic efficacy and decreasing systemic toxicity. However, the specific processes of the antibody delivery in vivo post injection are not known at the molecular level. Here we report the imaging of fluorescent nanoparticle conjugated with anti-HER2 antibody in living mice using a high speed confocal microscope. Semiconductor quantum dot (Qdot) are nanometer-sized crystals which improved brightness, resistance against photobleaching compared with organic dyes and fluorescent proteins. After intravenous injection of the tumor targeting quantum dot to living mouse with breast cancer, the accumulation of QDs to the tumor tissue was clearly observed at subcellular resolution with the original 3D intravital microscopic system. And we successfully track the movement of single particle of tumor targeting quantum dot in living mouse tumor tissue. This suggests that we can eventually develop a novel cancer imaging and drug tracking system. The tracking method used in our experiments is a powerful tool for developing drug delivery system to know where is a bottleneck in complex tumor microenvironments.

SP3
METACHRONOUS BILATERAL BREAST CANCER DISTINGUISHES BRCA1/2-POSITIVE FROM MUTATION-NEGATIVE FRENCH CANADIAN BREAST/OVARIAN CANCER FAMILIES
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Our group has shown that ~ 40% of French Canadian breast/breast-ovarian cancer families harbor germline BRCA1/2 mutations, where >80% are 1 of 5 recurrent mutations. Various mutation prediction programs were applied to a well characterized series of French Canadian cancer families (N=224) comprised of at least 3 cases of breast (Dx <65 yrs) and/or ovarian cancer occurring in 1st- , 2nd-, and/or 3rd-degree relatives of affected index case with known carrier status: 44 BRCA1-postive and 52 BRCA2-positive families, and 128

mutation-negative families. Using ROC analysis, the BRCAPRO model was the most accurate [C-statistic = 0.81, 95% CI 0.75-0.86], although the Manchester model incorporating information from 3rd-degree relatives modestly improved prediction scores. For the BRCAPRO model, a cut-off of 10% would have eliminated 60 of 128 (47%) mutation-negative families for genetic testing and only miss 10 of 96 (10%) mutation-positive families. At least one bilateral breast cancer case was observed in 71 of 224 (32%) families. While there was no significant difference in the number of bilateral cases in mutation-positive versus negative families, 70% (30/43) of the families with only metachronous bilateral breast cancer cases [p =0.01, 95% CI 0.54 to 0.83] occurred in mutation-carrier families. These results highlight the phenotypic differences in carrier-positive versus mutation-negative families as well as suggest improvements for the BRCAPRO model as this model does not take into consideration the phenotype of bilateral breast cancer.

SP4

AN INTEGRATED GENOMICS APPROACH TOWARDS THE IDENTIFICATION OF TUMOR SUPPRESSOR GENE CANDIDATES THROUGH NONSENSE-MEDIATED MRNA DECAY INHIBITION OF BREAST CANCER CELL LINES

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Our objective is to identify novel mutations in putative tumor suppressor genes (TSGs) using a new strategy based on the nonsense mediated decay (NMD) pathway. NMD is a protective cellular mechanism by which mRNA transcripts containing premature termination codons (PTCs) are degraded before the truncated proteins can be translated. Mutated transcripts containing PTCs can be stabilized in cells by pharmacologically inhibiting the NMD pathway through the use of Emitine. The accumulation of stabilized mRNA can be then monitored by comparing mRNA levels before and after treatment using Agilent cDNA expression microarrays. However this approach alone has identified a large number of stabilized messages possibly due to induction of stress response genes. As the inactivation of classical TSGs are due to somatic genetic events such as mutation of one allele and to deletion of the wild-type allele, we have focused our study on those candidates that map to the deleted chromosomal regions deduced by loss of heterozygosity (LOH) and comparative genomic hybridization (CGH) array analyses. Given the paucity of PTC mutations as mechanism of gene inactivation, we have also filtered our candidates by comparative analysis of multiple cell lines. As a final step in the process we filter those candidates that are up-regulated by Emitine treatment due to stress response. We are applying this approach to three human breast cancer cell lines (MCF-7, T47-D, and MDA-MB-231) Using this combined analysis 56 of 826 (7%) candidates were identified in the analysis of MCF-7. Some of the candidates identified in this approach have been independently associated with breast carcinogenesis. These candidates are currently being sequenced for validation. This combined integrated strategy which takes into consideration the principals behind classical inactivation of TSGs significantly reduces the number of candidates selected for sequence analysis.

SP5

IDENTIFICATION OF NOVEL GENES, GENE FAMILIES AND ONCOGENIC PATHWAYS INVOLVED IN MAMMARY CANCER BY MMTV INSERTIONAL MUTAGENESIS

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We performed a high-throughput retroviral insertional mutagenesis screen in MMTV-induced mammary tumors and identified 33 common insertions sites (CIS) associated with 17 novel candidate mammary cancer genes, 13 of which have never been linked to cancer. The human orthologs of these candidate cancer genes were also frequently deregulated in primary human breast tumors and associated with tumor parameters such as grade, angioinvasion and lymphatic infiltration. Computational analysis of all MMTV-tagged genes revealed specific gene families not previously associated with cancer, a significant overrepresentation of protein domains and signaling pathways associated with development and growth

factor signaling. Comparison of all tagged genes in MMTV and MuLV induced malignancies, showed that both viruses target mostly different genes acting predominantly in distinct pathways. Thus, exhaustive screening for MMTV-insertion sites reveals a novel repertoire of candidate breast cancer genes. In addition to the Wnt and Fgf genes, two genes, Rspo2 and Rspo3, belonging to the R-spondin gene family were among the most frequently tagged genes. All four members of the Rspo family encode secreted molecules with a single thrombospondin type 1 and two furin domains, but the function of these molecules has not been established. A subsequent RT-PCR study revealed that approximately 25% of all MMTV induced mammary tumors in BALB/c mice expressed one of the two former genes. In addition to Rspo2 and 3 also Rspo4 was frequently overexpressed in mouse mammary tumors relative to normal mammary epithelium, whereas Rspo1 was ubiquitously expressed in mammary tumors as well as normal mammary cells. Rspo2 and 3 were frequently activated in the same tumors that also overexpressed Wnt and Fgf genes suggesting that the Rspo genes collaborate with these oncogenes. Immortalized normal mouse mammary epithelial cells retrovirally transduced with Rspo3 cDNA were tumorigenic in nude mice, establishing Rspo3 as a novel oncogene.

SP6
LINKAGE DISEQUILIBRIUM OF GENES CODING FOR ROS METABOLISM AND SIGNALING, A COMPARISON OF LD PATTERN IN CASES AND CONTROLS

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Pharmacogenetic studies provide data of increasingly many single nucleotide polymorphisms (SNPs) in selected genes. Simultaneously, the HapMap project reveals the linkage disequilibrium (LD) map of all genes in different human populations. To what extent does this knowledge of the LD domains affect previous findings from pharmacogenetic studies of candidate SNP? Here we have selected candidate genes as part of a given functional pathway and report the extent of LD in 193 breast cancer patients and 86 cancer free Norwegian women. A total of 687 SNPs were successfully genotyped in both groups. Phase estimations were performed using PHASE v2.1, a program that implements methods for calculating haplotypes from population genotype data. Based on the PHASE output the extent of LD (D' and r2) and it's significance (Fishers exact test) were calculated. We observed strong LD in more than 70 genes (e.g. DPYD). rd We identified LD between SNPs in neighbouring clusters of genes, which have been previously studied in separate and independent studies (such as CCNB1 and XRCC4 (1q21) and CAT and GSTP1 (13p?). This notion of the existing LD is of potential value in designing new pharmacogenetic studies. Spearmans correlation coefficient was computed for each gene separately to show the correlation between the LD-values of cases and controls. For 33 genes (including NFKB1, PIK3CA, XRCC4 and TXNIP) a significantly different haplotype distribution was observed between cases and controls, indicating their potential importance in cancer development.

SP7
ROLE OF MATRIPTASE-2 (TMPRSS6) IN BREAST CANCER

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TMPRSS6 encodes for matriptase-2, a member of the type II transmembrane serine protease family that has been implicated in tumor progression. Expression of a close homologue of matriptase-2, matriptase-1 has been reported in breast and ovarian cancer.
We have previously reported chromosomal region 22q12-q13 and further a SNP rs733655 in matriptase-2 gene (*TMPRSS6*) to be associated with breast cancer. Here we report extensive case-control association analysis of the *TMPRSS6* gene by using 22 SNPs across the gene in an Eastern Finnish

population sample of 497 breast cancer cases and 458 controls. Genotyping was done using Taqman chemistry. In a total sample set marker rs733655 was significantly associated with breast cancer (p=0.044) and a 6-marker haplotype analysis of a 15kb region with high linkage disequilibrium showed a permutated p value of 0.001. In a more homogeneous subpopulation altogether 6 individual markers showed significant association with breast cancer with p-values ranging between p=0.05 and p=0.0002 and in a haplotype analysis with 8 markers a p-value of 0.01 was reached. Our results provide further evidence for *TMPRSS6* as a risk factor in breast cancer and urges for additional studies on the role of matriptase gene family in breast cancer.

SP8
SCREENING FOR MUTATIONS IN BREAST CANCER SUSCEPTIBILITY GENES

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Approximately 5-10% of breast and ovarian cancer cases are due to an inherited susceptibility gene mutation, especially in *BRCA1* and *BRCA2*. However, in breast cancer families the contribution of *BRCA1/BRCA2* germline mutations is only 10-20%. In 1-2% of the *BRCA1/BRCA2* mutation-negative cancer families, the disease appears linked to germline mutations in other known cancer susceptibility genes such as *TP53*, *PTEN*, *AR* and *ATM*, the predisposing gene still being unknown in all of the remaining cases.
BRCA2 has a specific role in DNA repair, regulating the activity of RAD51. The human *RAD51* gene is a homolog of RecA of *Escherichia coli* and functions in recombination and DNA repair. The interactions occur through conserved BRC repeats, encoded in *BRCA2*, binding directly to RAD51. The conserved TP53-binding protein 1 (53BP1) is a central mediator of the DNA damage checkpoint and appears to be one of the sensors of DNA DSBs. Improper processing of DSBs can result in loss or rearrangement of genetic information, leading to cell death or tumorigenesis. Based on the biological function of these genes, they are good candidates for being involved in cancer susceptibility.
In the current study patients belonging to 126 breast and/or ovarian cancer families were screened for alterations in the BRC repeats in *BRCA2*, *RAD51* and *TP53BP1* by using conformation sensitive gel electrophoresis (CSGE) and DNA sequencing.
A number of sequence variants were found, but none of them appeared to associate with cancer predisposition. The results of our study show that mutations in the BRC repeats in *BRCA2*, in the *RAD51* or *TP53BP1* gene do not play a major role in the initiation of breast cancer.

SP9
LARGE COLLABORATIVE STUDY OF THE BARD1 CYS557SER ALLELE: ENRICHMENT IN BRCA1/BRCA2 MUTATION-NEGATIVE BREAST CANCER FAMILIES

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Background: BARD1 was originally identified through its interaction with the breast and ovarian cancer susceptibility gene product BRCA1, with which it relates both structurally and functionally. BARD1 has been suggested to play a role both independently and in conjunction with BRCA1 in several functions associated with DNA repair and tumour suppression. Several studies have indicated that the *BARD1* gene is a potential target for germline changes predisposing to breast and ovarian cancer. The C-terminal Cys557Ser alteration has previously been uncovered to associate with an increased breast cancer risk and was recently shown to result in defectiveness in apoptotic activities.
Methods: Conformation sensitive gel electrophoresis (CSGE), minisequencing, Taqman assays, DHPLC analysis and DNA sequencing were used in order to investigate the prevalence of Cys557Ser in a large Nordic case-control study consisting of altogether

3956 breast and/or ovarian, prostate, colorectal and male breast cancer cases and 3591 controls.
Results: The frequency of Cys557Ser appeared to be increased among familial breast and/or ovarian cancer cases when compared to healthy controls (p=0.001).
Conclusion: Our results provide further evidence that *BARD1* Cys557Ser confers a slightly increased risk of breast cancer.

SP10
DETECTION OF SENTINEL LYMPH NODES BY NOVEL MR CONTRAST MEDIA

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Sentinel lymph node (SN) navigation surgery has been widely accepted as a method for made-to-order and low-invasive medicine. The size of particles is the most important factor for SN detection. We previously decided the suitable size for SN detection as 40nm by animal experiments. In this study, we aimed to clarify possibility of nano-sized particles of MR contrast media with appropriate size to make up disadvantages of existing methods like radioisotope or dye methods.
We developed nano-sized gadolinium beads in size of 40-60nm. We took MR imaging of the suspension of gadolinium nano-particles. And we injected the suspension of gadolinium beads into the rat's foot pad of the hind leg subcutaneously and followed MR scanning of the inguinal area for SN detection.
The nano-sized gadolinium beads exhibited higher signal intensity than water. It also showed changes of signal intensity in inguinal lymph nodes.
Existing contrast media for MRI passes so fast through lymph nodes that the timing of administration is difficult. As appropriate sizes allow nano-particles to stay at lymph nodes for a while, we could detect gadolinium nano-particles before and during surgery like radioisotopes. In conclusion, we claim that the nano-sized gadolinium beads have a potential to be an alternative to existing tracers for SN detection.

SP11
APPLICATION OF NANOTECHNOLOGY FOR BREAST CANCER RESEARCH: NANO-DDS AND MOLECULAR IMAGING BASED ON VISUALIZATION OF SINGLE PARTICLE IN VIVO

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Nanomedicine is the application of nanotechnology to prevention, diagnosis and treatment of human disease. It has a potential to change medical science dramatically in the 21st century. However, the research field is in its infancy, and it is necessary to grasp mechanism of pharmacokinetics, the toxicity on the occasion of application to medical treatment, in particular on the aspect of safety of the materials and devices. Here, we describe fluorescent nono-particles for sentinel node navigation for breast cancer surgery in experimental model, which have shown the potential to be an alternative to existing tracers in the detection of the sentinel node of if we select the appropriate particle size and wavelength. We also describe generation of CdSe nanoparticles, Quantum Dots (QDs) conjugated with monoclonal anti-HER2 antibody, Trastuzumab, for molecular imaging of breast cancer cells. The QDs-Trastuzumab complex coated with PEG was successfully made without decreasing the titer of antibody. We established a high resolution of 3D in vivo microscopic system as a novel imaging method at molecular level. The cancer cells expressing HER2 protein were visualized by the nanoparticles in vivo at subcellular resolution, suggesting future utilization of the system in medical applications including drug delivery system to target the primary and metastatic tumors. Future innovation in cancer imaging, not only at cellular level but also at molecular level, by synthesizing diagnostic agents with nanoparticles, is now expected.

SP13

LYSYL OXIDASE IS ESSENTIAL FOR HYPOXIA-INDUCED METASTASIS

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Metastases are responsible for the majority of cancer deaths and pose a particular problem in breast cancer as current treatment is ineffective for most detectable metastatic lesions in breast cancer patients. Metastasis is a multistep process that can be influenced by both the immediate microenvironment (cell–cell or cell–matrix interactions) and the extended tumor microenvironment (for example vascularization). Hypoxia (low oxygen) is clinically associated with metastasis and poor patient outcome, although the underlying processes remain unclear. Microarray studies have shown the expression of lysyl oxidase (LOX) to be elevated in hypoxic human tumor cells. Functionally, LOX initiates the covalent crosslinking of collagens and elastin in the extracellular matrix, increasing insoluble matrix deposition and tensile strength. We have found that LOX expression is regulated by hypoxia-inducible factor-1 and is associated with hypoxia in human breast tumors. Patients with high LOX expressing estrogen receptor negative tumors have poor distant metastasis-free and overall survivals (P=0.009 and P=0.015, respectively). Inhibition of LOX by shRNA, pharmacological inhibitors or by anti-LOX antibody eliminates metastasis in mice with orthotopically grown breast cancer tumors. Mechanistically, secreted LOX is responsible for the invasive properties of hypoxic human cancer cells through focal adhesion kinase activity and cell to matrix adhesion. Furthermore, LOX may be required to create a niche permissive for metastatic growth. Our findings indicate that LOX is essential for hypoxia-induced metastasis and is a good therapeutic target for preventing and treating metastases.

SP14

ISOLATION AND CHARACTERISATION OF NEW VARIANTS FROM BREAST METASTATIC MDA-MB-231 CELLS

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Our aim was to generate and characterise cell variants from metastatic MDA-MB-231 cells to find new molecular targets specific of the cell invasion. Using Boyden chamber coated with Matrigel we have selected in the presence of SDF-1□ chemokine two different populations of MDAMB-231 cells; MDA-MB-231-ref which did not invade the Matrigel and MDA-MB-231-inv which invaded the lower chamber within 24hrs and were obtained after four cycles of selection through the Matrigel. Stable MDA-MB-231-inv showed an invasion index two fold higher than the parental MDA-MB-231 and MDA-MB-231-ref cells. Characterisation showed that MDAMB-231-ref have pavement morphology whereas MDA-MB-231-inv are fusiforms. These different morphologies were conserved after several cell culture cycles and cell freezing. Doubling time of MDA-MB-231-inv (48 hrs) is twice more than those of parental MDA-MB-231 and MDA-MB-231-ref cells (24hrs). Since Neuropilin-1 (NRP-1) was shown to be involved in the MDAMB-231 cell motility (Bachelder et al. 2001) we have compared NRP-1 expression in these two MDA-MB-231 variant cells. MDA-MB-231-inv cells expressed 2 to 4 fold more NRP-1 than parental MDA-MB-231 and MDA-MB-231-ref cells, respectively. On the other hand, MDA-MB-231-inv cell adhesion on fibronectin was weak as compared to other cell types. Since MMP9 was shown to be directly correlated with the metastatic phenotype, we compared by zymography the MMP9 expression in these variants. MDA-MB-231-inv cells contained two fold more MMP9 than the other MDA-MB-231 cells. When inoculated subcutaneously in the mammary gland of nude mice, MDA-MB-231 parental cells tumour uptake was faster than the other types. After one month of tumour development, the volume and the mass of tumours were not significantly different for the three types of tumours.

Taken together these preliminary results constitute a new model to study the mechanism of metastatic process and suggest an implication of MMP9 and NRP-1 in the MDA-MB-231-inv phenotype.

SP15

A NEW PHENYL-BISPHOSPHONATE (BP7033) INHIBITS MDA-MB-231 AND MDA-MB-231-BO BREAST CANCER CELL INVASION BY INHIBITING RHO-CELL SIGNALLING PATHWAY AND CXCR-4 EXPRESSION

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In cancer therapy, accumulating evidence suggests that bisphosphonates have many biological effects beyong osteoclast inhibition. In the present study we have compared the effects of bisphosphonate Zoledronic acid (Zo) with a novel non containing-nitrogen bisphosphonate (BP7033) on the invasion of two type of metastatic cells: the MDA-MB-231 wild type ones and those which were isolated from bone . BP7033 (350nM) inhibited proliferation of MDA-MB-231 and MDA-MB-231 Bo in a similar manner while Zo had no effects on these two cell line However, Zo and BP7033 inhibited invasion through the Matrigel of the two cell lines with tha same IC50 (3.5nM).It is well known that invasion requires rho prenylation by Geranylgeranyl-diphosphate (GGPP) to lead Rho into the membrane and to activate Rock. Since invasion of both MDA-MB-231 and MDA-MB-231 Bo was completely reverted by GGPP but not by Farnesyl-phosphate (FPP) it seems that this novel bisphosphonate suppresses cell invasion by inhibiting Rho cell signalling pathway.Since CXCR-4 was shown to be involved in cancer metastasis,the effect of BP 7033 was also tested on CXCR-4 expression on the two MDA-MB-231 cell lines.

It was observed that MDA-MB-231-Bo cells express higher CXCR-4 expression than the corresponding wild type cells. CXCR-4 expression of MDAMB-231 was strongly inhibited by BP7033 at similar concentration obtained with Zo. CXCR4 of MDA-MB-231-Bo was 20 fold more inhibited than CXCR4 of MDA-MB-231.

Since BP7033 showed higher anti-proliferative effects than Zo and inhibited invasion via Rho signalling and CXCR4 expression inhibition, these results suggest that BP7033 could be a promising agent to use in anti-metastatic therapy.

SP16

STROMAL CELL-DERIVED FACTOR (SDF)-1: PREDICTIVE MARKER OF DISTANT METASTASIS AND POORER SURVIVAL IN BREAST CANCER

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SDF-1 is secreted by those organs to which breast cancer metastasizes, and serves to home in breast cancer cells that possess its receptor, CXCR4. Since SDF-1 is measurable in plasma, it was postulated that it could serve as a plasma marker for metastasis. In addition, an increased frequency of a polymorphism in the 3' untranslated region, SDF-1-3'A, was found in patients with breast cancer. Therefore, it was hypothesized that this polymorphism may be predictive of breast cancer metastasis. Plasma and lymphocytes were collected in 289 consecutive breast cancer patients with an average follow-up of 3 years. SDF-1 levels were measured in the plasma via an ELISA, and genotyping was performed via PCR-RFLP analysis. Patients were divided into low and high groups, based on the median plasma SDF-1 level. Of the patients who developed metastasis, there was an increased frequency of patients with low SDF-1 levels. Patients with low SDF-1 levels had significantly poorer breast cancer-specific and distant disease-free survival. Low SDF-1 level also appears to be an independent prognostic marker for distant disease-free survival. No association was identified between plasma SDF-1 levels and the SDF-1-3'A polymorphism. The polymorphism was associated with poorer overall survival and breast cancer-specific survival. Interestingly, patients with both low SDF-1 and the polymorphism demonstrated a much higher mortality rate (hazard ratio 3.2) compared to patients with low SDF-1 levels without the polymorphism. Therefore, it appears that low plasma SDF-1 levels may be the first host-derived blood marker predictive of breast cancer metastasis. The combination of low plasma SDF-1 levels and SDF-1-3'A polymorphism may together serve as strong prognostic factors in breast cancer patients.

SP17

THE INVASION SIGNATURE OF MAMMARY TUMORS IS CORRELATED WITH METASTATIC OUTCOME IN ANIMAL MODELS AND HUMANS

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The recent convergence of technologies for expression profiling and intravital imaging has revealed the identities of the genes involved in the survival, adjuvant-resistance and chemotaxis of invasive cancer cells inside living tumors. These genes fall into well defined pathways and are coordinately regulated in invasive tumor cells. This pattern is called the invasion signature. The invasion signature indicates that invasive cancer cells are a population that is neither proliferating nor apoptotic but highly chemotactic to macrophage-secreted EGF. In addition, the invasion signature predicts that invasive tumor cells, upon escaping the primary tumor, are non-dividing and resistant to chemotherapy, predictions that have been confirmed in vitro. Of particular relevance to hematogenous metastasis of breast tumors is the finding that the genes coding for pathways leading to amoeboid chemotaxis, i.e., the cofilin, Mena and ZBP1 pathways, are coordinately up-regulated in invasive cells collected from mammary tumors. Animal models in which the activity of the cofilin and ZBP1 pathways are individually elevated or decreased demonstrate that cofilin is a metastasis enhancer while ZBP1 is a metastasis suppressor. Mena expression is elevated in mice with spontaneous metastatic PyMT mammary tumors and in metastatic human breast tumors and tumor cell lines. Our results indicate that the expression status of these genes, either individually or in combination, will have significant value in the diagnosis of metastatic breast cancer.

SP18

THE DESIGN OF AN IN VITRO SCREEN TO IDENTIFY GENES MEDIATING LUNG METASTASIS USING A NON-INVASIVE ERBB2 MOUSE MODEL

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Breast cancer is leading cancer incidence and is the second leading cause of death in Canadian women. Although the 5-year survival rate is higher than 85%, half of the affected women will eventually die of complication from this disease such as metastasis. ErbB2, a receptor tyrosine kinase, demonstrates an increased activity in 20-30% of all cases of human breast cancer. We recently develop a ErbB2 knock-in model that mimics the genomic amplification and overexpression of ErbB2 seen in human breast tumors, although these mice rarely develop lung metastasis, suggesting that ErbB2 expression is not sufficient to drive tumor cell invasion. To take advantage of this feature and try to identify genes that are able to cooperate with ErbB2 to mediate lung metastasis, a functional in vitro invasion screen was designed, where cells derived from the knock-in model were infected with a retroviral cDNA library produced from mRNA extracted from polyomavirus Middle T (PyMT) mammary gland tumor. This in vitro screen yielded 21 full-length cDNA that were re-cloned into the retroviral vector and re-expressed into knock-in derived cells to confirm their pro-invasive ability. So far, 7 of the cDNA tested were able to promote invasion in vitro. Quantitative PCR confirmed that these invasive cDNAs are overexpressed 2-8 folds in PyMT tumors, while expression is very low in the knock-in mouse model. These cDNAs are currently being tested in an orthotopic mouse model to assess whether they are also able to promote lung metastasis in vivo. This screen will hopefully yield functional candidates that can act as prognosis markers and potential therapeutic targets for lung metastasis in human.

SP19
SAM68 NUCLEAR BODIES (SNBS) DYNAMICS IN CELL PROLIFERATION AND METASTATIC POTENTIAL
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Sam68 is a KH domain RNA binding protein whose RNA binding activity can be modulated by different post-translational modification such as arginine methylation, acetylation and phosphorylation. Sam68 is a known substrate of a member of the Src family of kinases, the Breast tumour kinase (BRK) and recently we showed that Sam68 becomes tyrosine phosphorylated, in a BRK-dependent manner, in response to epidermal growth factor (EGF) stimulation. Sam68 is predominantly nuclear and resides in Sam68 Nuclear Bodies (SNBs) in cancer cell lines. It was shown that both the RNA binding and the protein-interacting domain of Sam68 were required for its localization within SNBs. Here we show by gel filtration, that Sam68 resides within a large ribonucleoparticle complex (>1 MDa) in cells harbouring SNBs. RNAse treatment of the cellular extracts from BT-20 cells (derived from a breast tumour) shifted Sam68 to a smaller complex of ~200-450kDa. In cell lines lacking SNBs, Sam68 was present within the smaller complex and this correlated with greater cellular proliferation and metastatic potential, while presence of those SNBs decreased their proliferation and invasiveness potential. We observed that EGF stimulation (as well as PMA stimulation) could promote the disassociation of Sam68 within the large complex to form the smaller complex which also resulted in a significant decrease in the number of SNB positive cells and an increased cellular migration. The presence of these nuclear bodies and their cell cycle regulation suggest that Sam68 may play a role in cell proliferation and metastatic potential and could serve as a powerful prognostic tool to evaluate possible cancer recurrence.

SP20
CHARACTERIZATION OF THE ROLE OF STAT3 IN BREAST CANCER METASTASIS
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The 5 year survival rate for women with breast cancer drops from 98% in Stage I patients to 16% in patients at Stage IV. Breast cancer at Stage IV is characterized by metastases, thus the process of metastasis is closely linked to breast cancer mortality. We have focused on Stat3, a protein putatively involved in the metastatic process. Stat3 is a member of the signal transducers and activators of transcription family and acts downstream of a variety of receptor types including receptor tyrosine kinases such as ErbB2. The phosphorylated or active form of Stat3 has been linked to metastasis of a number of cancer cell types. Stat3 has also been implicated in metastasis through its transcriptional regulation of a number of pro-metastatic factors including matrix metalloproteinases (MMPs). In activated ErbB2 and polyomavirus middle T (PyVMT) tumours we have shown a high level of expression of activated Stat3 both by immunoblot and immunohistochemical analyses. Stat3 phosphorylation was also detected in lung metastases by immunohistochemistry. We have looked at the role of Stat3 *in vivo* by breeding $Stat3^{flx/flx}$ mice with mice harboring Cre recombinase under the control of the mouse mammary tumour virus (MMTV) promoter. The resulting $Stat3^{flx/flx}$/MMTV-Cre mice were characterized at different stages of mammary gland development using wholemount analyses. These mice have also been interbred with mice expressing PyVMT under control of the MMTV promoter. MMTV-PyVMT mice exhibit short tumour latencies and nearly 100% of this strain develop metastases in the lungs. $Stat3^{flx/flx}$/MMTV-Cre/MMTV-PyVMT mice were monitored for both tumour development and metastatic status in order to assess the effect of Stat3 knockout in the context of a highly metastatic mouse strain.

SP21
THE STRUCTURE/FUNCTION RELATIONSHIP OF LIMK1 IN BREAST CANCER MIGRATION AND INVASION
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One of the main areas of investigation in the Gutierrez-Hartmann lab is the study of tumorigenesis and metastasis in breast cancer. Recently, LIM-kinase 1 (LIMK1) has become a key focus of investigation for its role in promoting

metastatic behavior in models of human cancer. My work aims to define the structure/function role of LIMK1 in migratory and invasive behavior of breast cancer.

To this end, a series of LIMK1 constructs is currently being utilized to map the relationship between the functional domains of LIMK1 and the phenotypic migratory and invasive behavior observed in cells that overexpress LIMK1. Additionally, shRNA is being employed to alter expression levels of LIMK1, as well as the expression levels of key effectors of LIMK1. A system consisting of MDA-MB-231 cells, and a derivative line of these cells that were selected in nude mice to be highly metastatic, serve to model the structure/function relationship of LIMK1 in breast cancer.

A series of LIMK1-expressing retroviruses encoding LIMK1 truncations and fusions with distinct subcellular targeting sequences have been constructed. These viruses are being employed to systematically correlate the functional domains of LIMK1 to the migratory and invasive processes underlying LIMK1's role in metastasis. The functional outcome of alterations in LIMK1 activity and subcellular localization is being assessed via scrape wound assays and matrigel invasion assays. Additionally, nude mice mammary injections will be used to measure in vivo metastasis. Together, these studies should provide a comprehensive and detailed analysis of the precise role of LIMK1 in breast cancer motility and metastasis, and thus also provide an additional therapeutic target molecule to treat breast cancer.

SP22
N-CADHERIN SIGNALING POTENTIATES MAMMARY TUMOR METASTASIS

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N-cadherin (N-cad) is upregulated in breast carcinomas with aggressive or metastatic phenotype, but its mechanism of action in vivo remains unknown. We generated transgenic mice coexpressing N-cad and Polyoma virus Middle T antigen (PyMT) in the mammary epithelium. In these mice, there was a substantial increase in pulmonary metastasis with no differences in tumor onset or growth compared to control PyMT mice. PyMT-N-cad tumor extracts exhibited increased ERK, P38 MAPK and Akt phosphorylation relative to PyMT controls. P-ERK staining was enhanced in pulmonary metastasis and was upregulated in PyMT-N-cad mice compared to controls. Mammary tumor cell lines derived from PYMT-N-cad mice expressed high constitutive levels of p-ERK, displayed enhanced motility, invasion and MMP-9 secretion relative to cells derived from PyMT tumors. Treatment of PyMT-N-cad cells with low concentrations of the MEK1 inhibitor, U0126, reduced MMP-9 production and cellular invasiveness (but not motility), while P38 inhibition had no effect on these events. These results demonstrate that *denovo* expression of N-cad in the transformed mammary epithelium exacerbates the metastatic phenotype of breast tumors, via enhanced P-ERK signaling, leading to MMP-9 production, invasion and metastasis.

SP23
ESTRADIOL ENHANCES THE IL-3 INDUCED GROWTH STIMULATION OF BONE-METASTATIC BREAST CARCINOMA CELLS

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Bone is the most common distant site of metastasis in breast cancer patients. Distant metastases offer a dismal 10% survival for breast cancer patients. Only recently the molecular mechanisms involved in the early establishment of bone metastases had begun to be elucidated. Our laboratory had previously shown that exposure of bone-metastatic breast carcinoma cells to estradiol enhances the growth-stimulatory effect normal human bone marrow conditioned medium. In addition, we showed that IL-3 is a critical growth factor for bone-metastatic disease. Recently, we identified Jak-2 as an intracellular mediator of the IL-3- induced growth of bone-metastatic cells. Based on this information we hypothesized that estradiol enhances the growth of bone-metastatic cells by inducing activation of Jak-2. To test this hypothesis we exposed bone-metastatic MDA-231 breast cancer cells to IL-3 (150 ng/ml) for 24-48 hrs. The MDA-231 bone-metastatic cells were grown in estradiol and estradiol-free conditions. After protein

extraction, immunoblotting was performed using commercially-available antibodies for Jak2 and p-Jak-2. After chemoluminescence, the bands were digitalized and normalized using β-actin as control. We found that estradiol enhances the phosphorylation of Jak-2 after IL-3-stimulation of bone-metastatic. Stimulation of bone-metastatic cells with IL-3 in the absence of estradiol does not induced activation of Jak-2. These findings suggest that estradiol play a major role in the IL-3-mediated growth of bone-metastatic breast carcinoma cells.

SP24
IDENTIFICATION OF INVASION SPECIFIC SPLICE VARIANTS OF THE CYTOSKELETAL PROTEIN MENA IN MAMMARY TUMOR CELLS

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We have studied the gene expression pattern of invasive primary breast cancer cells by utilizing a unique in vivo invasion assay which isolates the invasive cells by chemotaxis. One of the genes up regulated in the invasive cells is Mena, an actin binding protein involved in the regulation of cell motility and adhesion. In mouse there are five splice variants and in humans there are three known splice variants of Mena. Mena expression is regulated during breast tumor progression. Mena is over expressed in high risk benign lesions, primary and metastatic tumors. Using the in vivo invasion assay in rats and mice with mammary tumors we observed that two isoforms of Mena up regulated in the invasive tumor cells. RT-PCR and QRT-PCR studies confirmed this over expression. Sequencing and sequence alignment studies were conducted revealing a high level of homology between the mouse invasion isoforms for Mena. From the sequence alignment we have identified a unique region (+++) in the splice transcripts of the invasion isoform which is present in both human and mouse tumor cells. Antibodies generated against this unique region will allow us to identify metastatic cells in tumors and therefore predict patient outcome.

SP25
THE CONTRIBUTION OF HOST-DERIVED TGF-BETA 1 TO BREAST CANCER OUTGROWTH IN BONE

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Interactions between metastatic tumor cells and bone-derived growth factors facilitate the growth of tumor cells in this unique microenvironment. TGF-β1 is a cytokine that is stored in abundance within the bone matrix and can be released and activated during osteoclast-mediated bone resorption. Furthermore, MDA-MB-231 breast cancer cells rely on TGF-β signaling for efficient metastasis to bone. Thus, it is believed that the bone matrix represents a reservoir of TGF-β that can promote the formation of breast cancer induced osteolytic lesions in bone. To investigate a role for host-derived TGF-β1 in promoting breast cancer outgrowth in bone, we utilize mice homozygous for a null mutation in TGF-β1 (TGF-β1-/-; Rag2-/-). Our breast cancer cell model is an *in vivo* selected MDA-MB-231 bone metastatic population tagged with Green Fluorescent Protein (GFP) and Firefly luciferase (Fluc) that expresses low levels of latent TGF-β1. When injected directly into the tibia, MDA-MB-231 #1833 GFP/Fluc cells formed osteolytic lesions with the greatest frequency in TGF-β1+/+; Rag2-/- animals followed by TGF-β1+/-; Rag2-/- mice. Interestingly, TGF-β1-/-; Rag2-/- mice had the lowest incidence of osteolytic outgrowth of all three genotypes.
One caveat of this genetic approach is functional compensation for TGF-β1 loss by the continued presence of TGF-β2 and TGF-β3, which are both also present in bone. To address this concern, we have begun to characterize a soluble TGF-β inhibitor composed of the extracellular domain of the TGF-β type II receptor fused to two distinct dimerizing motifs (RII/E+K coils). Conditioned media from MDA-MB-231 parental cells expressing the RII/E+K coils efficiently blocks TGF-β1 responsiveness in epithelial cell lines (Mv1Lu and MNuMG). Importantly, these effects are not observed in MDA-MB-231 cells harboring empty vector controls. Currently, RII/E+K coil and empty vector MDA-MB-231 cells are being tested

following intra-tibial injection for their ability to form osteolytic lesions *in vivo*.

SP26
BREAST CANCER METASTASIS TO BONE: A ROLE FOR OSTEOACTIVIN

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Bone is the number one site of breast cancer metastasis; these metastases are often osteolytic and can result in bone pain, increased risk of fracture, hypercalcemia, and reduced quality of life. Therapeutic strategies to eliminate bone metastasis have been largely ineffectual due to an incomplete understanding of this complex, multi-step process. To address this issue we have utilized 4T1 cells, a murine mammary carcinoma cell line, with the capacity to metastasize to bone from the mammary fatpad of Balb/c mice. Parental 4T1 (4T1p) cells were injected orthotopically into Balb/c mice and produced radiologically detectable osteolytic bone metastases in 33% of mice. Bone Metastatic (BM) 4T1 cells were flushed from the bone marrow, expanded in culture and serially re-injected into mice. Utilizing this approach, we have isolated 4T1 sub-populations which will form osteolytic lesions in 70-80% of mice following orthotopic injection. We have performed microarrays (Agilent platform) on the parental and several in vivo selected, aggressive BM sub-populations of 4T1 cells and have identified osteoactivin (OA) as a gene that is expressed at higher levels in the aggressive BM populations. We have confirmed this increase in OA expression by northern blot and immunoblot.
OA is a type I transmembrane glycoprotein with extracellular heparin binding and integrin recognition motifs. It is expressed at high levels in many human cancers and can promote cell motility and invasion. Additionally, OA is expressed in dendritic cells and has been proposed to mediate trans-endothelial migration of these cells by facilitating adhesion to endothelial cells. We will show that our in vivo selected, OA expressing BM populations are significantly more motile, invasive, and adherent to components of the extracellular matrix. Efforts to functionally implicate OA in these processes through siRNA knock-down and over-expression studies in vitro will be discussed.

SP27
LOCALIZATION AND EXPRESSION OF KEY CELL-CELL AND CELL-MATRIX ADHESION PROTEINS IN PRIMARY CULTURE MODELS OF BREAST CANCER INVASION

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Background/Aims: In breast cancer, little is known of whether ductal carcinoma in situ (DCIS) can progress to invasive ductal carcinoma (IDC). Loss or redistribution of specific adhesion proteins may be involved in the acquisition of invasive characteristics. By generating primary cell cultures from patients with DCIS and IDC tumours, our aim was to assess the mechanistic involvement of cell-cell and cell-matrix adhesion proteins in a potential transition from in situ to invasive carcinoma. Confocal microscopy and western blotting were used to assess the localization and expression of key adhesion proteins in two- and three-dimensional cultures in the presence or absence of EGF treatment.
Results/Conclusions: We have successfully grown several primary cell cultures from DCIS and IDC biopsies. Our preliminary results show greater expression of the cell-matrix adhesion protein CD44 in DCIS cells treated with EGF. Furthermore, we observed some disrupted localization of the cell-cell adhesion protein ZO-1 in DCIS and IDC cells in comparison to a normal breast epithelial cell line MCF-10A. Our ongoing work is to establish the mechanistic importance of such phenomena in these valuable translational models.

SP28
DISCOVERY AND VALIDATION OF A PROMISING NEW TARGET FOR THERAPEUTIC MONOCLONAL ANTIBODIES: THE IMMUNOMODULATORY PROTEIN B7-H4 IS OVEREXPRESSED IN HUMAN BREAST AND OVARIAN CANCERS

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Through genomics efforts we identified an mRNA encoding a cell surface protein (DD-O11O) that is overexpressed in human breast and ovarian

cancer with low/no expression in normal tissues. Immunohistochemical studies with antibodies against DD-O11O revealed strong tumor cell surface staining in a majority of human ductal and lobular breast adenocarcinomas, breast cancer metastases and serous ovarian epithelial cancers. We further showed that DD-O110 protein is extensively glycosylated and displayed on the surface of cultured breast tumor cell lines. Other groups recently identified a new member of the B7 immunomodulatory protein family, B7-H4, which is identical to DD-O110. B7-H4 protein is expressed on the surface of a variety of activated immune cells and functions as a negative regulator of T cell responses. Thus overexpression of B7-H4 by human cancers may inhibit an anti-tumor immune response. We have obtained additional data for a functional role of B7-H4 in cancer showing that this protein can promote tumor formation when overexpressed in tumor epithelial cells. We generated monoclonal antibodies which recognize native human DD-O110 protein and bind efficiently to the surface of live human tumor cell lines as well as to human breast or ovarian cancer samples. Antibodies bound to B7-H4 on tumor cells can effectively internalize. The restricted normal tissue distribution of B7-H4, its over-expression in a majority of breast and ovarian cancers together with a functional role in promoting tumor growth make this cell surface protein an ideal target for a monoclonal antibody therapeutic strategy. Efficacy studies with a set of promising monoclonal antibodies are in progress.

SP29

AN ASSESSMENT OF THE INTERACTION OF MITOMYCIN C AND DOXORUBICIN IN EMT6 MOUSE MAMMARY TUMOR CELLS

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The co-application of the chemotherapeutic agents mitomycin C and doxorubicin has been shown to result in supra-additive tumor cell killing *in vitro* and *in vivo*. Here, we determined if this interaction was truly synergistic and defined the parameters that enabled the intracellular interaction of these drugs. The median effect analysis (MEA) was used to determine the degree of interaction, as it is free from bias and mechanistic assumptions. To determine the parameters important for this interaction, formaldehyde and reactive oxygen species formed from drug metabolism were measured, and DNA cross-linking and DNA double-strand breaks were followed as genotoxic endpoints. In addition glutathione levels were modulated, and major metabolizing enzymes (i.e. DT-Diaphorase and CYP450) were inhibited in whole cells. The interaction of mitomycin C and doxorubicin was found to be a true synergy. *In vitro* evidence identified glutathione levels in the cell as an important mediator of this interaction, and thus mitomycin C-derived products of metabolism (e.g. formaldehyde, mitomycin C reactive metabolites) that are known to deplete glutathione as potential players. Since DNA cross-links were found to only increase additively with co-administration of the drugs, we propose that the poisoning of topoisomerase II-alpha by doxorubicin interacts with the drug-induced DNA cross-links. Therefore, the synergy observed between mitomycin C and doxorubicin may be elicited through mitomycin C-derived formaldehyde and reactive metabolites, glutathione depletion and the enhanced genotoxicity of the combination of DNA cross-links and topoisomerase II-alpha poisoning.

SP30

NANOSIZED SILVER IODIDE BEADS AS NEW CONTRAST MEDIA FOR SENTINEL LYMPH NODE NAVIGATION SURGERY

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The objective of this study was to evaluate the possibility of the new nanosized silver iodide beads (AgI) with silica coating as a X-ray contrast medium. Silica coating was performed to prevent acute and fatal reactions. We tried to apply AgI for Sentinel Lymph Node Biopsy (SNB). Some institution reported the effectiveness of X-ray CT method for identifying sentinel lymph node (LN). But traditional contrast media go through LN so quickly that it is diffucult to detect LN precisely.As the size of beads determines staying and passing time in the LN, we can control enhancement period by modifying the size of beads. We also assessed its temporal distribution with X-CT after subcutaneous and intravenous AgI injection to a rabbit. The contrast-enhancement of a blood vessels, lymph

nodes, the liver and the spleen was observed. Compared with existing contrast media, AgI showed delayed washing out. We can carry out SNB with AgI beads in most institutions which have X-ray equipment and can detect sentinel lymph node more precisely than radio isotopes before surgical treatments. In addition, AgI nanoparticles can prevent allergic reactions by silica coating. AgI are expected to be employed in clinical fields by further examination in the future.

SP31
IN VIVO ROLE OF THE SIX1 HOMEOPROTEIN IN MAMMARY GLAND TUMORIGENESIS

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Human Six1 is a homeodomain-containing transcription factor that is critical for the expansion of precursor populations during development. In addition to its developmental role, overexpression of Six1 has been detected in a number of human cancers, including breast cancer, where it is linked to both proliferation and metastasis. As many as 50% of primary breast cancers and 90% of metastatic lesions overexpress the gene, in part due to gene amplification. Six1 can transform a mammary epithelial cell line, but no work has been done to show the effects of Six1 overexpression *in vivo*. We have established an inducible, mammary-specific Six1 overexpression model by crossing MMTV-rtTA mice to TetO-Six1 mice, and are using this model to test whether Six1 overexpression leads to mammary tumors, as well as to dissect the molecular mechanism by which Six1 influences tumorigenesis *in vivo*. Data from this model are suggestive of both a developmental and tumorigenic phenotype. Overexpression of Six1 throughout pregnancy and lactation leads to an increased frequency of litter loss and decreased pup weight, suggesting that Six1 overexpression impairs lactation and normal mammary differentiation. In mice induced to overexpress Six1 in the mammary gland for 5 months, a hyperprolifative phenotype is observed. Furthermore, preliminary data indicate that invasive mammary adenocarcinoma is observed with long latency and constitutive Six1 overexpression. Data from this model suggests that inappropriate expression of Six1 impairs differentiation and promotes tumorigenesis. This inducible model provides us with a system to examine whether removal of Six1 expression can reverse the phenotypes, thereby addressing whether Six1 is a viable drug target. Importantly, Six1 is not necessary for most normal adult tissues, and thus therapies directed against Six1 may not lead to the severe side effects seen with more conventional treatments.

SP32
IDENTIFICATION OF TARGET GENES MEDIATING GROWTH INHIBITION BY THE TRANSCRIPTION FACTOR C/EBP DELTA

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The transcription factor C/EBPdelta promotes apoptosis of mammary epithelial cells during mammary gland involution. Furthermore, overexpressed C/EBPdelta inhibits the growth of certain human breast tumor, prostate tumor and leukemia cell lines. In the mouse mammary gland, C/EBPdelta promotes mammary epithelial cell apoptosis during post-lactational involution. The direct downstream targets of C/EBPdelta mediating growth arrest and/or apoptosis in breast epithelial cells however are unknown.
Cyclin D1 is an important regulator of the cell cycle and frequently overexpressed in breast tumors cells. Emerging evidence suggests that cyclin D1 might also act through pathways other than as a cell cycle regulator. Given its importance in tumorigenesis, understanding the transcriptional regulation of the cyclin D1 promoter may help in the identification of molecular targets.
Our data suggest that cyclin D1 expression is regulated by C/EBPdelta in breast epithelial cells. Overexpression of C/EBPdelta in mammary epithelial cells resulted in down regulation of cyclin D1 levels. By chromatin immunoprecipitation assay, EMSA and reporter assays, we identified at least one element in the cyclin D1 promoter that is targeted by C/EBPdelta. Thus, our data suggest that C/EBPdelta promotes growth arrest and apoptosis in part via the down regulation of cyclin D1 promoter.

SP33
DEVELOPMENT OF DNA/POLYCATION COMPLEXES ENABLING MORE EFFICIENT GENE TRANSFECTION TO BREAST CANCER CELLS FOR GENE THERAPY

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In the murine experimental systems, tumor cells transfected *in vitro* with various genes induced anti-tumor effects when injected into mice. We have demonstrated that mouse hepatoma MH134 cells transfected with FasL gene implanted into mice were rejected after inducing infiltration of neutrophils [1]. Importantly, the mice that had rejected tumor cells acquired antitumor immunity against non-transfected MH134 tumors and when FasL-transfected and non-transfected hepatoma cells were mixed and injected into mice, both cells were rejected through the mechanism of anti-tumor immunity. For application of the results to gene therapy of human breast cancer, however, it is necessary to transfect the target gene directly to cancer cells *in vivo*. For this purpose, we are developing DNA/polycation complexes that enable more efficient transfection of genes to breast cancer cells using murine mammary tumor, BJMC3879 (BJMC) and MH134 cells *in vitro* and *in vivo*. Polyethyleneimine (PEI) as a polycation was investigated for transfection of FasL gene. To augment the efficiency of the transfection, cDNA plasmid with PEI was modified with polyethyleneglycol (PEG) and/or JTS-1, as DNA/polycation complexes. The result revealed that the plasmid modified with both PEG and JTS-1 was the best complex by Luciferase assay and X-gal staining in both tumor cells (MH134 and BJMC) in vitro. On the other hand, since PEG induced aggregation with serum albumin *in vivo* due to cationic charge, we tried to modify PEG with carboxylic acid or succinate [2, 3]. FasL mRNA production was observed by RT-PCR. Moreover, we are investigating the efficiency of transfection with Luciferase assay and planning the gene therapy using FasL gene. DNA/PEI complex modified with PEG-succinate and JTS-1 would be a useful tool for gene therapy of breast cancer.

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SP34
THE ROLE OF SHORT CDP/CUX ISOFORMS IN CANCER

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The CDP/Cux transcription factor plays a role in cell cycle progression. The full-length protein of 200 kDa is proteolytically processed by nuclear cathepsin L at the G1/S transition into an isoform of 110 kDa (p110). A second isoform of 75 kDa (p75) is generated from an alternative mRNA. The p110 and p75 isoforms are overexpressed in different types of cancers, such as in leiomyomas and breast cancers. In tissue culture, p110 can accelerate cell proliferation by activating the G1/S transition. In the present study we investigated the oncogenic potentials of both the p75 and p110 isoforms. We have found that proteolytic processing of CDP/Cux is increased in many transformed cells and was no longer cell cycle regulated. Cysteine protease expression and activity correlated with the extent of CDP/Cux processing. Oncogenic ras caused an increase in transcription and translation of cathepsin L leading to the production of short nuclear cathepsin L isoforms and enhanced processing of CDP/Cux. A cell-permeable cysteine protease inhibitor was able to delay progression of ras-transformed 3T3 cells into S phase and their proliferation in soft-agar. Furthermore, overexpression of the processed CDP/Cux isoform was able to stimulate cell motility and invasion. To investigate the oncogenic potential of the short CDP/Cux isoforms, we engineered transgenic mice expressing either p200, p110 or p75 under the control of the mouse mammary tumor virus long terminal repeat, specifically integrated by homologous recombination into the hprt locus. 33% of the p75 CDP/Cux mice and 30% of the p110 mice developed a malignancy. While some of the tumors arose from the mammary gland, most of the tumors in virgin females originated from various tissues and cell types. Overall, these results confirm the

oncogenic potential of CDP/Cux p75 and p110 and reveal a specific spectrum of tumor types for each isoform.

SP35
DIFFERENTIAL EFFECTS OF CORE HISTONES ON THE INHIBITION OF PROLIFERATION OF HUMAN BREAST CANCER CELLS

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Introduction: Inhibitors of histone deacetylase (HDAC) are effective anticancer agents. HDAC inhibition results in the accumulation of acetylated histones, including the core histones H2A, H2B, H3, and H4.
Objectives: The present study was designed to assess whether exogenous core histones arrest cancer cell growth. As our histone source contains the acetylated form of histones, we proposed that the treatment of cancer cells with exogenous histones may elicit a direct anticancer effect, similar to that observed with HDAC inhibition.
Methods: The MCF-7 human breast adenocarcinoma cell line, as well as additional cell lines were treated exogenously with core histones and their effects on cell growth, anchorage independence, cell cycle, molecular markers, as well as the underlying mechanism were analyzed.
Results: Utilizing the MCF-7 cell line, we demonstrate that histone H2A decreases the proliferation of MCF-7 cells. We observed similar findings with the HCC1806 human breast cancer and the NXS2 murine neuroblastoma cell lines. Histone H2A treatment of MCF-7 and NXS2 cells leads to cell cycle arrest in the S and G2/M phases, and markedly reduces its capacity for anchorage-independent growth. The treatment of MCF-7 cells with exogenous histone H2A induces cellular senescence, which is characterized by an increase in senescence-associated ß-galactosidase (SA-ß-galactosidase), senescence-associated changes in cell morphology, and irreversible growth arrest. Histone H2A-treated MCF-7 cells upregulate the cell cycle inhibitor $p21^{WAF1/CIP1/Sdi1}$, while p53 protein levels are unchanged. In contrast, histone H2B, H3, and H4 were ineffective in the inhibition of human breast cancer cell proliferation using our experimental conditions.
Conclusions: In spite of the common features found in all core histones, histone H2A is unique amongst the core histones in its capacity to inhibit human breast cancer cell proliferation. Future studies will aim to identify which properties of histone H2A underly cancer cell arrest.

SP36
NOVEL RETINOID 9-CIS UAB30 DOWN-REGULATES HTERT AND THE DNA METHYLTRANSFERASES IN BREAST CANCER CELLS WITH REDUCED TOXICITY

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Retinoic acids and their derivatives potentiate anticancer effects in breast cancer cells. The aberrant expression of telomerase is critical to the continued proliferation of most cancer cells. Thus, telomerase is an attractive target for chemoprevention and treatment of breast cancer. 9cUAB30 is a novel synthetic retinoid X receptor-selective retinoic acid (RA) that effectively reduces the tumorigenic phenotype in mouse breast carcinoma with lower toxic effects than natural retinoid treatments. We have assessed 9cUAB30 retinoic acid treatment of three estrogen receptor-positive human breast cancer cell lines to determine the potential of this drug as an effective telomerase inhibitor and its application to cancer therapy. 9cUAB30 was found to inhibit proliferation and decrease telomerase and DNA methyltransferase expression in MDA-MB-361, T-47D, and MCF-7 breast cancer cells. 9cUAB30 also reduced colony formation in soft agar assays in each of these cell types. Combination treatments of 9cUAB30 and all-trans RA proved to be even more effective than either RA alone, suggesting a possible epigenetic mechanism in the anti-telomerase activity of the retinoids. Therefore, the novel retinoid, 9cUAB30, is effective in inhibiting the growth of human breast cancer cells and its anticancer mechanism may be related to telomerase inhibition mediated through epigenetic modifications.

SP37
IDENTIFICATION OF DOWNSTREAM TARGETS OF ESTROGEN AND C-MYC IN BREAST CANCER CELLS

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Estrogen plays a pivotal regulatory role in the control of cell proliferation in the normal breast and breast cancer but the target genes through which estrogens regulate cell proliferation and the abnormalities in this pathway that result in loss of normal growth control and differentiation in breast cancer are not fully understood. Transcriptional activation of c-Myc is an early event in estrogen-induced mitogenesis and inducible expression of c-Myc can over-ride antiestrogen-induced growth arrest, implying that overexpression of c-Myc or its target genes could modulate sensitivity to endocrine therapies. Consistent with this hypothesis, induction of c-Myc conferred resistance to ICI 182,780, tamoxifen and raloxifene. To identify targets of c-Myc and estrogen with likely roles in proliferation control, we compared transcript profiles of antiestrogen-arrested cells stimulated to re-initiate cell cycle progression by estrogen treatment or c-Myc induction. Approximately 2/3 of the probesets significantly regulated by estrogen (adjusted $p < 0.01$) increased in expression. Half of the estrogen-regulated probes were also regulated by c-Myc and more than 20% of the probes that increased in expression were known estrogen- or c-Myc targets. Analysis of selected candidates has identified a direct target of c-Myc whose expression is correlated with c-Myc expression in breast cancer. Genes involved in ribosome biogenesis and protein synthesis were over-represented in the probes increased by both estrogen and c-Myc, while genes involved in proliferation were over-represented overall. These data suggest a model in which estrogen regulates ribosome biogenesis predominantly via c-Myc but regulates proliferation only partially via c-Myc.

SP38
ESTROGEN AND PROGESTERONE RECEPTORS AS TARGET FOR THE THERAPY OF BREAST CANCER - RECENT ADVANCES, MECHANISMS OF RESISTANCE, AND NEW APPROACHES

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While the first antihormones, CPA and Tamoxifen were found rather by chance, deeper understanding of nuclear receptors as transcriptional factors enabled more rational structure-activity based drug discovery. We describe the pharmacological characterization of a novel, highly potent progesterone receptor (PR) antagonist, that has a considerable potential not only for therapeutic intervention in breast cancer but rather may prevent this disease more efficiently. The biological activity of progesterone is mediated by the PR, which induces a cascade of transcriptional events, critical for maintenance and development of female reproductive organs. Blocking PR function by using a PR-antagonist allows the modulation of various endocrine diseases.
The PR-antagonist showed high antiprogestagenic activity in vitro on both PR isoforms PR-A and PR-B. Subsequent experiments with breast cancer models showed a strong antiproliferative activity. In the NMU and DMBA-induced mammary tumor models in the rat, treatment with the PR-antagonist completely supressed the growth of established tumors and prevented the development of breast tumors when given prophylactic. The ability of this compound to induce tumor cell apoptosis is unique among all other endocrine therapeutics. Our results revealed that the biological response to a PR-antagonist does not seem to be only the result of competition of progesterone but rather may be accompanied by additional mechanisms. With these pharmacological properties a PR-antagonist may be a promising new option for clinical breast cancer therapy. Results from a drug-finding program on pure antiestrogens will be reported. These new steroidal antiestrogens are highly active pure estrogen receptor antagonists with an efficient degradation of the estrogen receptor protein without any agonistic activity. Data obtained in preclinical tumor models in mice and rat showed an intrinsic potency in growth inhibition of estrogen receptor positive breast cancer.

SP39

EXPRESSION REGULATION OF ER-ALPHA BY ESR1-PROMOTOR C METHYLATION AND PROGNOSTIC VALUE OF ESR1-ISOFORM C FOR THE SURVIVAL OF BREAST CANCER PATIENTS

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Estrogen receptor alpha (ERα) positivity in breast cancer patients (BCP) indicates a favorable disease outcome in the assessment of risk for progression and benefit for endocrine therapy. ERα is the Tamoxifen target, but it is not sufficient to predict treatment success. One-third of BCP are ERα-negative ab initio, and among ERα-positive patients up to 40% will relapse and die. Paraffin-embedded BC tissue samples of 138 patients diagnosed between 1988-1999 were retrieved from Pathology Department. ERα-status was established by immunohistochemistry. DNA/RNA were extracted from punched tumor areas. Methylation-specific-PCR and bisulfite-restriction-analysis were used for qualitative evaluation of methylation patterns at ESR1-promoters A and C. Using MALDI-TOF the percentage of methylation was calculated at specific CpG promoter C sites. Quantitative-Real-Time-PCR was used to measure transcript levels of ESR1: isoform ESR1-C (promoter C driven), ESR1-A/C (promoters A or C driven). Promoter C methylation was inversely correlated with ERα-status while promoter A methylation was not. Particularly promoter C methylation at CpG+99 was correlated with receptor loss (p=0,00). Among patients with ERα-positive tumors Kaplan-Meier analysis revealed a significant decrease of overall survival for cases over-expressing ESR1-C with an associated risk for recurrence or cancer related death (OR=5.24 95%CI 1.06-25.96). ESR1-C expression was confirmed as prognostic factor for overall survival in Cox-regression when stage, tumor size, nodal status, grading were included as covariates (HR=2.29 95% CI 1.06-4.94). We demonstrated a role of promoter C regulation on ERα loss with striking evidence for the CpG+99 site methylation being a potential marker for ERα negativity. Moreover, ESR1_C isoform levels were of prognostic value with respect to survival of tamoxifen treated BCP.

SP41

EFFECT OF MUC1 EXPRESSION ON EGFR ENDOCYTOSIS AND DEGRADATION IN HUMAN BREAST CANCER CELL LINES

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EGFR is a member of the ErbB family of tyrosine kinase receptors its overexpression has been tied to tumor formation in the mammary gland. Upon ligand binding, EGFR homo or heterodimerizes with other members of the ErbB family and is the endocytosed. MUC1 is a large transmembrane glycoprotein that is overexpressed in 90% of breast cancers. Overexpression of MUC1 in the breast induces the formation of metastatic tumors. Our preliminary data shows that MUC1 and EGFR interact in breast cancer cells but not in the normal polarized mammary epithelium.

We hypothesize that by modifying the cellular trafficking of EGFR and affecting its degradation, MUC1 is enhancing EGFR signaling. This interaction will then promote EGFR-dependent proliferation and inhibition of apoptosis, thereby promoting tumor formation.

We show that MUC1 knockdown in BT20 breast cancer cells induces faster degradation of ligand-bound EGFR compared to MUC1-expressing cells. Localization of endocytosed EGFR demonstrates that it localizes to a perinuclear compartment of unknown identity where internalized MUC1 resides. We also show through biotinylation of surface proteins that MUC1 affects the degradation of ligand-bound EGFR. Surprisingly, MUC1 increases the rates of EGFR internalization but does not appear to affect the recycling of EGFR. This suggests that MUC1 is affecting EGFR trafficking in a way that stabilizes EGFR intracellularly. Future experiments will focus on dissecting the effect of MUC1 overexpression on the endocytic pathway of EGFR. We will also investigate how MUC1 overexpression affects Cbl, Grb2 and CALM association with EGFR during endocytosis (key proteins in the endocytosis and degradation of EGFR). Finally, we will determine what part of the MUC1 cytoplasmic domain is responsible for the effects on EGFR endocytosis and degradation.

SP43

TARGETING TACE-DEPENDENT EGFR LIGAND SHEDDING IN BREAST CANCER

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The ability to proliferate independently of signals from other cell types is a fundamental characteristic of the tumor cell. Here, we analyze a model of human breast cancer progression in which this transition has occurred and delineate the mechanism by which growth factor signaling autonomy has been established. In this model, proliferation of the malignant cells, "T4-2", is driven by an autocrine loop not present in non-malignant, "S1", cells. This loop is upregulated at the earliest stage of progression toward malignancy in this model (S2) and consists of two growth factors, Amphiregulin and TGFa. We show that TACE/ADAM17 activity is required for the function of these growth factors in both normal and malignant cells and that inhibition of this protease results in downregulation EGFR pathway activity and phenocopies Iressa-induced EGFR inhibition by reverting the malignant phenotype of T4-2 cells in a 3D culture assay. Although TACE has many substrates, we demonstrate definitively that the reverting effect of TACE inhibition is a direct consequence of the inhibition of growth factor ectodomain shedding and show that TACE inhibition elicits a transcriptional profile similar to inhibition of both EGFR and MEK. We show that TACE-dependent growth factor shedding is prevalent in breast cancer cell lines and can be effectively targeted by TACE inhibition. We further demonstrate a strong positive correlation between TACE and TGFa expression in human breast cancers that is predictive of poor prognosis. These data implicate TACE as a therapeutically tractable enzyme, the inhibition of which effectively blocks EGFR signaling by preventing mobilization of ligands for this receptor and suggest that co-ordinate inhibition of TACE may augment the activity of EGFR inhibitors in a clinical setting.

SP44

VEGF-RECEPTOR-2 AND PLEXIN-A1 ARE CO-EXPRESSED WITH NEUROPILIN-1 IN BREAST CANCERS

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Neuropilin-1 (NRP-1) is a membrane receptor binding vascular endothelial growth factor-165 (VEGF165) and class 3 semaphorins (SEMA3) in a competitive fashion. NRP-1 forms a complex with VEGF165 and co-receptor VEGF-receptor-2 (VEGFR-2), mediating cell survival, invasion and migration. The competitive binding of NRP-1 to SEMA3s requires the co-expression of receptors plexins-A. NRP-1 expression is documented in breast cancer cell lines, but not in breast tumor samples. We investigated NRP-1 protein expression in epithelial cells of breast tissue samples together with expression of VEGFR-2, VEGF, plexinA1, SEMA3A, VEGFR-1, on a tissue-microarray (TMA). The TMA contained 16 benign breast lesions, 12 in situ carcinomas, 107 invasive breast cancers. Cytoplasmic staining intensity was evaluated with a semi-quantitative scoring (0 for negative, 1+/2+/3+ for weak to strong positive). Almost all invasive breast tumors showed some staining for NRP-1: 38% scored as 1+, 33% as 2+, and 28% as 3+. Conversely, no benign lesion was 3+, while 50% were negative (p=2+ for VEGFR-2 while 40% were positive for plexinA1. When receptor co-expression was evaluated, almost all VEGFR-2-expressing tumors were also positive for NRP-1 (>=2+). Similarly, almost all plexinA1 positive tumors were positive for NRP-1 expression. The expression of all tested proteins increased significantly (Spearman's test) from benign lesions to invasive cancers. SEMA3A staining was >=2+ in only 13% of invasive cancers, almost always in the presence of plexinA1 >=2+. These findings suggest that NRP-1 may be an active co-receptor for VEGFR-2 and/or plexinA1 in about 50% of breast tumors. The staining for NRP-1, VEGFR-2 and plexinA1 is being evaluated for correlations with prognosis on a larger breast prognostic TMA.

SP45

HER-2 GENE AMPLIFICATION BUT NOT CHROMOSOME 17 POLYSOMY DEFINES A DISTINCT BREAST CANCER PHENOTYPE

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Apart from HER-2 gene amplification, it has also been suggested that polysomy 17 could lead to HER-2 overexpression in breast cancer. So far, it remains unclear whether polysomic 17 tumors share biological characteristics with truly HER-2 amplified tumors. In the present study, a series of 198 invasive breast carcinomas showing HER-2 overexpression by immunohistochemistry (IHC 2+/3+), were subjected to dual-probe FISH analysis. In addition, cases were examined by IHC for estrogen (ER) and progesterone receptor (PgR) expression. We further linked our findings to axillary lymph node status, patient's age at diagnosis and histopathological features of the tumor. All 87 (100 %) IHC 3+ and 30 out of 111 (27.0 %) IHC 2+ scoring cases showed HER-2 gene amplification by FISH. Polysomy 17 without HER-2 gene amplification was found only in IHC 2+ scoring cases (in 44.2 %). An ER-/PgR- phenotype was found more frequently in HER-2 amplified tumors (40.2 %) compared to HER-2 non-amplified polysomic 17 (4.1 %) or HER-2 normal (6.25 %) tumors. ER+/PgR+ tumors with concomitant HER-2 gene amplification were characterized by young age at diagnosis (median age 47), high frequency of lymph node metastases (58.8 %) and poor tumor differentiation. We therefore suggest that triple positive ER+/PgR+/HER-2 amplified tumors might represent a distinct clinico-pathological entity. Finally, our data indicate that polysomy 17 is of minor importance in HER-2 overexpressing breast cancer as compared to HER-2 gene amplification.

SP46

BRCA1/2 ASSOCIATED KOREAN BREAST CANCER HAS MORE OFTEN ER/C-ERBB2 NEGATIVE PHENOTYPE

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Purpose: Although incidence rate of female breast cancer is still low in Korea, its incidence has been rising quite rapidly. An earlier age of onset of Korean patients than in Western countries suggests that it would be related with genetic background. The prevalence of BRCA mutation in Korean breast cancer is estimated to be about 2.8%~3.1% for sporadic and 11.3%~12.7% for high-risk patients. The purpose of this study was to evaluate clinicopathologic and biologic features of BRCA mutation-associated breast cancer in Korea.
Methods: The study subjects consisted of 262 patients at high-risk of BRCA mutations. Germline mutations in the entire coding sequences of the BRCA1,2 genes were analyzed by Conformation-Sensitive Gel Electrophoresis (CSGE) method, and any aberrantly-sized bands were sequenced. Of total patients, 20 patients had mutations (13 BRCA1 mutations and 7 BRCA2 mutations). We compared BRCA mutation group with no-BRCA mutation group, retrospectively.
Results: The BRCA mutation group had higher proportion of ER negative (70.0% vs 34.8%, p=0.002), higher proportion of high histologic grade (63.2% vs 40.3%, p=0.05), higher proportion of family history (55.0% vs 35.1%, p=0.076), and lower proportion of c-erbB2 overexpression (10.5% vs 24.8%, p=0.26), compared to the no-BRCA mutation group. ER/c-erbB2 negative or triple negative (ER/PR/c-erbB2 negative) is more often in BRCA associated breast cancer (42.1% vs 13.4%, p=0.004; 36.8% vs 12.6%, p=0.019, respectively).
Conclusions: BRCA1/2 associated Korean breast cancer have high proportion of ER negative and ER/c-erbB2 negative, which is characteristics of basal-like breast cancer.

SP47

MAMMARY-SPECIFIC RON RECEPTOR OVEREXPRESSION INDUCES HIGHLY METASTATIC MAMMARY TUMORS ASSOCIATED WITH β-CATENIN ACTIVATION

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Activated growth factor receptor tyrosine kinases play pivotal roles in a variety of human cancers, including breast cancer. Ron, a member of the Met receptor tyrosine kinase protooncogene family, is overexpressed or constitutively active in 50% of human breast cancers. To define the significance of Ron overexpression and activation in vivo, we generated transgenic mice that overexpress a wild type or constitutively active Ron receptor in the mammary epithelium. In these animals, Ron expression is significantly elevated in mammary glands and leads to a hyperplastic phenotype by 12 weeks of age. Ron overexpression is sufficient to induce mammary transformation in all transgenic animals and is associated with a high degree of metastasis, with metastatic foci detected in liver and lungs of over 86% of all transgenic animals. Furthermore, we show that Ron overexpression leads to receptor phosphorylation and is associated with elevated levels of tyrosine phosphorylated β - catenin and the upregulation of genes, including cyclin D1 and c-myc, which are associated with poor prognosis in patients with human breast cancers. These studies suggest that Ron overexpression may be a causative factor in breast tumorigenesis and provides a model to dissect the mechanism by which the Ron induces transformation and metastasis.

SP48

ANTIBODY AGAINST EPITHELIAL MUCIN (MUC1) FROM BREAST TUMOR-INFILTRATING B LYMPHOCYTES

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Human mucin MUC1 is one of the well-characterized tumor antigens expressed in adenocarcinomas of breast, pancreas, lung, colon, ovaries and prostate. Analysis of the immune response in patients with such tumors revealed the presence of low-titer anti-MUC1 antibodies in their sera. We explored the capacity of patient's immune system to produce anti-MUC1 antibodies in tumor microenvironment by analyzing tumor-infiltrating B lymphocytes. We demonstrated that 70% of primary breast tumors (7 out of 10) contains oligoclonal antibody repertoire, indicating the significance of spontaneous humoral immune response inside tumor tissue. We generated several scFv recombinant antibody libraries from primary tumor-infiltrated B lymphocytes and panned the mixture of the libraries against recombinant MUC1 protein containing 5.3 tandem repeats of the protein core. The isolated novel anti-MUC1 single-chain antibody, MB5, recognized the target protein in ELISA test. The binding capacity of MB5 was assessed by immunofluorescence staining of tumor cells and also measured by flow cytometry. The MB5 intensively stains MCF7 cells, known for high MUC1 expression, and also reacts with another tested breast carcinoma cell line, SkBr3. For further improvement of affinity of the selected antibody, a secondary maturation scFv library was constructed. After selection in stringent conditions, more reactive anti-MUC1 single-chain antibodies were isolated. In this study we demonstrate that tumor samples obtained as discarded surgical material can be used as appropriate source for recombinant phage display libraries enriched for tumor-specific antibodies. Isolation of anti-tumor scFv toward the MUC1surface antigen shows this approach to be very promising in developing the tumor-targeting antibodies potentially useful for diagnosis and treatment of cancer.

SP49

FROM GENES TO DRUGS: BIN1 AND IDO IN IMMUNE ESCAPE AND THERAPY OF BREAST CANCER

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Immune escape is a key feature of cancer progression about which little is known.

Indoleamine 2,3-dioxygenase (IDO) suppresses T cell activation and its broad activation in human cancers may facilitate immune escape. However, it is not known how IDO becomes elevated in cancer nor whether IDO inhibitors may be useful to treat established tumors. We recently identified IDO as a genetic target of inhibition by Bin1, a cancer suppression gene that is attenuated in many human breast cancers and other malignancies. Studies of human clinical cases and a Bin1 knockout mouse model support the hypothesis that Bin1 loss facilitates cancer progression, in part by facilitating IDO-mediated escape from T cell-mediated immunity. On the basis of these observations, we identified bioactive small molecule inhibitors of IDO and performed a preclinical evaluation of their potential anticancer activity. In MMTV-neu mice, a well-established transgenic model of breast cancer, IDO inhibitors can cooperate with cytotoxic chemotherapy to elicit regression of tumors that are refractory to single-agent therapy. Notably, as little as 4-5 days of co-treatment with an IDO inhibitor is sufficient to produce regressions at a 2 week endpoint. Based on these and other findings, clinical translation of a lead inhibitor is currently being pursued in collaboration with the NCI and NewLink Genetics Corporation. Our findings suggest that Bin1 suppresses cancer in part by limiting the ability of IDO to suppress T cell-dependent immune surveillance. They also illustrate how IDO inhibitors can be used to safely enhance the ability of cytotoxic chemotherapy to destroy breast tumors. General implications for combining chemotherapy and active immunotherapy will be discussed.

SP50
EFFECTS OF RETINOID ON MAMMARY GLAND DEVELOPMENT AND BREAST CANCER

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Background: Because puberty represents a key time during which breast cancer risk is determined, adolescent mammary gland development has been identified as a target for chemopreventive strategies. Epidemiological data identified chemopreventive capabilities of retinoid. Consistent with these data, retinoid are anti-proliferative and pro-differentiative in cell culture models—attributes anticipated to reduce breast cancer risk. Contradictory to these data are clinical trials, in which retinoid treatment promoted lung cancer in high risk individuals. *Methods:* We investigated the effects of adolescent Vitamin A intake on susceptibility of the adult gland to MNU-induced carcinogenesis in Sprague-Dawley rats. Rats were administered diets deficient, adequate or mildly supplemented with retinoid from 21 to 63 days of age, during which mammary gland development occurs. Following the 42-day dietary intervention, rats were administered adequate levels of retinoid for the duration of the study, limiting the intervention to adolescence. Rats were carcinogen treated at 70 days of age.
Results: In our studies, retinoid supplementation had inconsistent effects on breast cancer risk, but consistently inhibited mammary gland alveolar development. It was not anti-proliferative or pro-apoptotic, as in vitro work predicted. We assayed the effects of retinoid on progesterone, a hormone required for alveolar differentiation—circulating progesterone levels were unaffected by retinoid intake. Progesterone receptor expression was also unchanged in response to retinoid. However, retinoid did correlate to decreased expression of the progesterone-responsive genes WAP, β-casein, and CBP/p300, suggesting that retinoid supplementation reduces progesterone-responsive gene transcription. Interestingly, retinoid supplementation correlated with decreased expression of C/EBP, which is thought to work in conjunction with the progesterone receptor to promote alveologenesis. *Conclusions:* Adolescent mammary gland development is highly modulated by dietary intake of retinoid, and impacts susceptibility to chemical carcinogens in the adult. The implications of these data for breast cancer prevention will be discussed.

SP51
TAMOXIFEN TREATMENT FUNCTIONALLY ALTERS THE RAT MAMMARY STROMA, INDICATING A ROLE FOR THE EXTRACELLULAR MATRIX IN TUMOR SUPPRESSION

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The functional unit of cancer has been described as the tumor cell plus its microenvironment because specific interactions between the tumor cell and the extracellular matrix (ECM) can either promote or suppress metastatic spread of the cancer. Thus, we

propose that the epithelial and stromal compartments are altered by interventions such as tamoxifen treatment that reduce breast cancer risk and in fact, changes in both compartments are required for the observed efficacy. We tested the hypothesis that tamoxifen functionally alters the mammary stroma in a rat model. As expected, tamoxifen treated rats were found to have mammary glands with a more ductal phenotype, indicative of a reduced epithelium, and less proliferative mammary epithelial cells than control rats. Primary mammary fibroblasts harvested from tamoxifen treated animals had reduced cell motility. In addition, we observed changes in the composition of ECM molecules such as decreased FN fragments and MMP activity in tamoxifen animals. We then isolated ECM from pooled mammary glands. These ECM preparations were tested for ability to support organoid formation in 3D culture and were used as substratum in transwell filter motility and invasion assays. Human mammary epithelial MCF-12A cells transformed by the V12 Ras oncogene displayed a very aggressive 3D phenotype when plated on control matrix, characterized by a lack of strong cell-cell associations and numerous filipodia locally invading into the matrix. However, on the mammary matrix isolated from tamoxifen treated animals, these same cells formed multi-cellular organized structures without invasive filipodia. Highly aggressive MDA-MB-231 cells also showed a less aggressive 3D phenotype on 'tamoxifen matrix'. Both cell lines displayed reduced motility or invasiveness in the presence of 'tamoxifen matrix'. These data indicate that tamoxifen treatment functionally alters the mammary stroma and provides support for the role of the microenvironment in mediating suppression of tumor progression.

SP52
MINOSPHERES-PREMALIGNANT MAMMARY TISSUE CULTURE FROM THE MINO MOUSE MODEL OF HUMAN DCIS

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Breast cancer progresses from morphologically defined pre-invasive proliferations (DCIS) to invasive carcinoma, but the mechanisms and markers of progression are elusive. We have developed and characterized serially transplantable mammary intraepithelial neoplastic outgrowth lines (MINO) derived from FVB Tg(MMTV-PyVmT) mice, a molecular mimic of human ERBB2 amplified breast cancer. We have characterized the tumor latency, tumor incidence, and pulmonary metastasis in these MINO lines. In this study, we sought to optimize the transfer of normal, premalignant (MINO) and invasive tumor mammary tissue to non-adherent suspension cultures and test the biology of these cultured tissues by retransplantation. Dissociated and cultured normal, and MINO tissues produce organized spheroids. The MINO phenotype and biology are retained after retransplantation into the gland cleared fat pad. Spheroids were disassociated and grown in Matrigel at limiting dilutions. Single or small cell groups proliferate to form new spheroid "mammospheres". The morphology and organization of MINO spheroids closely resemble those derived from normal mammary glands. These "MINO-spheres" form disorganized clusters of cells along one pole rather than a branching morphology. Ki67 immunostain confirms an increased proliferation in these foci, predominantly with a keratin 8/18 luminal phenotype but with occasional SMA/K14 positive cells present in the disorganized clusters. This MINOsphere model allows in vitro transfection of normal and premalignant mammary tissue to elucidate which molecular pathways are necessary or sufficient for malignant transformation and analyze physiological aspects. The MINOspheres are amenable to techniques developed for in vitro physiologic analysis. Single cell dissociations permit the isolation of population of putative "stem" cells to analyze the potential of a given cell phenotype to recapitulate premalignant mammary tumor progression.

SP53
ROLE OF NOTCH SIGNALING IN CELL-FATE DETERMINATION OF MOUSE MAMMARY STEM/PROGENITOR CELLS

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The Notch gene family is a frequent target for insertional mutagenesis by the mouse mammary tumor virus (MMTV) in mouse mammary tumors. Our lab has recently shown that over-expression of Notch1 and Notch3 intracellular domain ($Notch1^{IC}$ or $Notch3^{IC}$) mutant in Tg mice blocks mammary gland development and induces tumors. Considering the well-known role of Notch signaling in

maintaining stem cell characteristics in the hematopoietic, neural, intestinal system and pancreatic cells, the mammary tumor potential of Notch members has raised the possibility that the precisely regulated self-renewal of mammary epithelial stem and progenitor cells is mediated by Notch signaling and is subverted in tumor cells to allow malignant proliferation. Tumors may originate from the transformation of normal stem cells, the so called "cancer stem cells", a population of rare cells within the tumors with indefinite potential for self-renewal and which has the potential to drive tumorigenesis. Therefore, we hypothesize that Notch1^{IC} or Notch3^{IC} mutant affects mammary stem cell lineage commitment, thus leading to the transformation of normal stem cells. Our results show that `mammary gland stem cells` can been isolated from normal or Tg mice by their expression properties of cell surface marker CD24 and CD29 and form a distinct population definable by flow cytometry. This normal lin-CD24^{+} CD29^{high} display multi-differentiation potential in vitro. These normal, but not Notch1IC Tg, CD24^{+} CD29 ^{high} cells form mammary gland like structures and express cytokeratin three days after seeding in vitro in the present of matrix component. Transplantation of these normal lin-CD24^{+} CD29^{high} into cleared fat pad generates an entire mammary gland. Further comparison of growth and differentiation of non-Tg and Notch1^{IC} (and Notch3^{IC}) Tg stem cells in vitro and in vivo (cleared fat pads) will be presented. These studies should enable us to gain insight into general molecular mechanisms of mammary cell-fate control.

SP54
DIFFERENTIAL GENE EXPRESSION PROFILE OF CARCINOMA ASSOCIATED FIBROBLASTS AS COMPARED TO THOSE DERIVED FROM NORMAL BREAST TISSUE BY SERIAL ANALYSIS OF GENE EXPRESSION

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The homeostasis of normal breast depends on interactions between epithelial cells and their associated stroma. Previous studies indicated that in breast cancer carcinoma, tumor associated stromal cells with different functions appear to be emerged. These stromal elements include fibroblasts which modulate tumor behavior providing various growth factors and extracellular matrix components. Our aim was to evaluate the differential gene expression between carcinomas associated fibroblasts (CAF) and normal breast associated fibroblasts (NAF) using Serial Analysis of Gene Expression (SAGE). After establishment of primary cultures, fibroblasts were subjected to immunocytochemical evaluation in order to verify the purity of culture. The RNA was then extracted and SAGE was performed. A total of 136,492 tags was generated by sequencing. 61,130 from CAF (20,424 unique tags) and 75,362 (31,849 unique tags) in NAF, between these tags, 14,205 and 20,305 have annotation in CAF and NAF respectively; 6,900 tags were found differentially expressed in both libraries, using a 4 fold changes cut-off, 605 genes were hypoexpressed and 399 hyperexpressed. A large number of genes involved in different cellular processes, such as proliferation, adhesion and cytoskeleton organization, were differentially expressed in both libraries, showing intrinsic alterations in CAF transcriptome, since we could verify a higher number of genes whose expression was downregulated in CAF, which might be due to epigenetics mechanisms.

SP55
METABOLIC MICROENVIRONMENT IN MOUSE MAMMARY PRECANCERS

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As first described by Warburg, tumors exhibit increased uptake of glucose and anaerobic glycolysis. The former allows tumor growth assessment using Positron Emission Tomography (PET). MicroPET images of mouse mammary tumors and precancers support the notion these tissues have a unique metabolic microenvironment. Our studies of the molecular and metabolic events occurring in precancers and their tumors might provide a mechanistic explanation.

Mammary tumors, mammary intraepithelial neoplasms (MINO) (precancers) and outgrowth lines from MIN (MINO) obtained from mice engineered with polyoma virus middle T (Tg(PyVmT) were used. MicroPET imaged both MINO and tumors. The tissues were studied using expression microarrays. The hypoxia-related gene Carbonic anhydrase XII was upregulated 124-fold in MINO and tumors as compared to normal mammary gland. Tumors stained for Carbonic Anhydrase 9, a hypoxia-related marker, but MINO stained heterogeneously with higher concentrations in clusters of atypical cells.

Carbonic anhydrases bind to and modulate the sodium-hydrogen exchanger (NHE1). NHE1, a ubiquitous protein, modulates internal pH (pHi). As previously, western blots show NHE1 levels are elevated in Tg(PyVmT) tumors but not normal mammary gland or MIN. Consistent with past observations in glioma cells, baseline pHi of tumors was alkaline (7.27) relative to normal mammary epithelium (6.93) when measured in vitro using BCECF. MINO cells pHi were relatively acidotic (6.73). As predicted from known NHE1 behavior, recovery of pHi following NH4 washout was proportional to the degree of intracellular acidification.

These data suggest precancers differ metabolically from normal and invasive cancer cells. The transition into invasive disease may involve the selection of a subset of precancer cells that can compete successfully in and, in part, create an acidotic extracellular environment.

SP56

INVOLVEMENT OF TUMOR DERIVED-FACTORS IN THE MODULATION OF NFKB AND C/EBP EXPRESSION IN MACROPHAGES FROM TUMOR BEARING MICE

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Interactions between malignant tumors and the host immune system shape the course of cancer progression. The molecular basis of such interactions is the subject of immense interest. Previous work from our laboratory has shown that macrophages from mice bearing the D1-DMBA-3 mammary tumor are profoundly dysfunctional, exhibiting decreased levels of IL-12 and nitric oxide. We have demonstrated that two major transcription factors involved in the functions of the innate immune system and the inflammatory response, NFkB and C/EBP, show decreased binding activities to their respective promoter sites on the IL-12p40 and iNOS genes in macrophages from tumor-bearing mice. We have recently found that the deficient binding activities of these two transcription factors is associated with their reduced expression and diminished nuclear translocations in these cells. These functional dysregulations may represent a manifestation of a more general phenomenon, by which tumor-derived factors contribute to tumor evasion, interfering with the expression and function of key transcription factors. An analysis of several tumor-derived factors secreted by the DA-3 cell line isolated from the D1-DMBA-3 tumor was undertaken. It was found that TGF-b1 and PGE2 , but not VEGF, downregulate the expression of these two transcription factors. We propose that tumor-derived factors may operate as modulators of NFkB and C/EBP expression, two transcription factors that are crucial for an adequate immune response, providing a novel mechanism of tumor evasion.

SP57

BREAST CANCER PATIENTS EXPRESSING B7-H1 MOLECULE IN THEIR TUMOR CELLS SHOW HIGH MITOTIC INDEX

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The B7-H1 is a negative co-stimulatory molecule that can induce apoptosis in T-lymphocytes resulting in an immune escape of tumors. This molecule is expressed in many types of cancer including breast cancer. We have shown previously a strong association between B7-H1 expression in breast cancer patients and the presence of high histologic grade as measured by the standard Scurf-bloom-Richardson (SBR) scoring system. In the present work, we have subjected the B7-H1 expression in 69 breast cancer patients against the 3 compounds (tubular differentiation, nuclear pleiomorphism, and mitotic index) of the SBR. A strong and significant correlation was observed with the mitotic index (P= 0.007) indicating an association between B7-H1 expression and cancer cell proliferation. The

association between B7-H1 expression and proliferation was further investigated in breast cancer tissues immunohistochemically against the standard proliferation marker Ki-67. We have shown a highly significant correlation between B7-H1 and Ki-67 expression ($P<0.001$).

The relation of B7-H1 induction and cell proliferation was further investigated in vitro using primary cultured cells. B7-H1 was induced in 8/8 primary cultured cell lines (50 to 100 % expression) and its induction was gradual ranging from 7% on day 1 to 100% on day 10. This expression was accompanied with a parallel increase in cell proliferation as measured by Ki-67 staining. Furthermore B7-H1 was downregulated in cells arrested in the G0 phase confirming the involvement of the proliferation factor in the induction of B7-H1. In conclusion, we have shown a strong association between B7-H1 expression in breast cancer patients and a high proliferative index. The induction of B7-H1 in proliferative breast cancer cells may contribute to the bad prognosis associated with highly proliferating tumors. Understanding the mechanisms by which B7-H1 is induced in proliferative cancer cells may help in designing specific therapeutic drugs for breast cancer.

SP58
COOPERATION BETWEEN INTRACELLULAR RAS ACTIVATION AND THE MICROENVIRONMENT LEADS TO IMMORTALIZATION OF HUMAN MAMMARY EPITHELIAL CELLS

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Normal human fibroblasts have been shown to senesce in response to oncogenic Ras, while initiated cells have been shown to undergo transformation. We have previously identified a rare subpopulation of variant human mammary epithelial cells (vHMEC) that display some characteristics of tumor cells, suggesting that they have engaged the process of malignant transformation. Therefore, we hypothesized that like initiated cells, vHMEC would be resistant to Ras-induced senescence. To test this hypothesis, we introduced constitutively active Ha-RasV12 into vHMEC (vHMEC-Ras). Consistent with the idea that vHMEC are already engaged in the transformation process, vHMEC-Ras failed to undergo a proliferative arrest as seen in normal fibroblasts expressing oncogenic Ras, and displayed many chromosomal abnormalities. Since the microenvironment can modulate characteristics of epithelial cells, we tested the possibility that extracellular signaling could cooperate with intracellular Ras activation in altering the behavior of vHMEC. To mimic the secretory aspects of the extracellular environment, we exposed both vHMEC carrying control vector and vHMEC-Ras to media containing serum. We found that extracellular stimulation resulting from the presence of serum was sufficient to cause immortalization of vHMEC-Ras but not control vHMEC. Cells immortalized with Ha-RasV12 displayed an increase in telomerase activity and the capacity for anchorage independent growth. These cells are currently being tested for their ability to form tumors when injected orthotopically into the mammary fat pads of mice. Our data are consistent with previous observations that multiple insults are required for the transformation of primary human mammary epithelial cells, and suggest that such insults may be intrinsic or extrinsic.

SP59
EVALUATING PROTEASES IN POPULATIONS OF PRIMARY HUMAN MAMMARY EPITHELIAL CELLS ISOLATED FROM AREAS OF HIGH OR LOW MAMMOGRAPHIC DENSITY

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Women with breast tissue of high mammographic density are at higher risk for developing breast cancer. Areas of high mammographic density contain greater amounts of total collagen and therefore we analyzed proteases implicated in collagen turnover in populations of mammary epithelial cells (HMEC) isolated from patient biopsies of either high mammographic density (HDAE) or low mammographic density (LDAE). HMEC were grown on reconstituted basement membrane (rBM) or collagen I. On rBM, both HDAE and LDAE formed structures resembling branching terminal end buds. When grown on rBM for 48 hr in a live cell assay for degradation of dye

quenched (DQ)-collagen IV, LDAE exhibited enhanced collagen IV degradation, suggesting they might differ in expression of gelatinases. Gelatin zymography however did not reveal differences in MMP-2 and -9 expression. Secretion of MMP-2 was observed when cells were grown on rBM, but not on collagen I. Since cysteine cathepsins degrade collagens endocytosed by the uPARAP pathway, we performed the DQ-collagen IV assay in the presence of an activity-based probe for cysteine cathepsins. This assay revealed co-localization of active cysteine cathepsins and degraded DQ-collagen IV. Furthermore, LDAE expressed higher levels of the cysteine cathepsins B and L than did HDAE. Increases in cathepsin B and L were observed by immunoblotting when HMEC were cultured on rBM, but not on collagen I. Our results are consistent with the cysteine cathepsins participating in degradation of collagen IV by LDAE and HDAE, perhaps contributing to the low mammographic density associated with the LDAE.

SP60

TRANSFORMATION OF DISTINCT HUMAN BREAST EPITHELIAL CELL TYPES LEADS TO DIFFERENT TUMOR PHENOTYPES

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The influence of cell-of-origin on solid tumor phenotype is poorly understood. We investigated this aspect of malignant phenotype by comparing tumors derived from two distinct normal human mammary epithelial cell (MEC) populations, one of which was isolated by using a novel culture medium. Transformation of these two cell populations through the introduction of the same set of genetic elements resulted in cells that formed tumor xenografts showing major differences in histopathology, tumorigenicity, and metastatic behavior. While transformation of one epithelial cell type (HMECs) yielded squamous cell carcinomas, a rarely occurring human tumor in the human breast, transformation of the other epithelial cell type (BPECs) yielded tumors closely resembling human adenocarcinomas. Transformed BPECs were 10,000-fold more tumorigenic and gave rise to lung metastases, unlike non-metastatic HMECs. We conclude that the pre-existing differences between these two normal breast cell populations (cells-of-origin) strongly influence the phenotypes of their transformed derivatives, including their tendency to metastasize. Interestingly, these experiments also revealed that much (>90%) of the tumor-cell gene expression profile is dictated by the nature of the normal cells-of-origin. Majority of the genes that were altered with transformation in one cell type (HMEC or BPEC) either remained unchanged or were altered in the reverse direction in the other cell type. Hence, isolation and biological analyses of specific normal cell populations that give rise to different tumor types will be essential for a complete understanding of tumor phenotypes and therapeutic responses.

SP61

HER2/NEU (ERBB2) SIGNALING TO RAC1-PAK1 IS TEMPORALLY AND SPATIALLY MODULATED BY TRANSFORMING GROWTH FACTOR-BETA

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In HER2 (ErbB2)-overexpressing cells, TGF-beta, via activation of phosphatidylinositol-3 kinase (PI3K), recruits actin and actinin to HER2, which then colocalizes with Vav2, activated Rac1 and Pak1 at cell protrusions. This results in prolonged Rac1 activation, enhanced motility and invasiveness, Bad phosphorylation, uncoupling of Bad/Bcl-2, and enhanced cell survival. The recruitment of the HER2/Vav2/Rac1/Pak1/actin/actinin complex to lamellipodia was abrogated by actinin siRNAs, dominant-negative (dn) p85, gefitinib, and dn-Rac1 or dn-Pak1, suggesting that the reciprocal interplay of PI3K, the HER2 kinase, and Rac GTPases with the actin cytoskeleton is necessary for TGF-beta action in oncogene-overexpressing cells. Thus, by recruiting the actin skeleton, TGF-beta crosslinks this signaling complex at cell lamellipodia; this prolongs Rac1 activation and increases metastatic properties and survival of HER2-overexpressing cells.