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BASIC SCIENCE

Dominant-Negative Histone H3 Lysine 27 Mutant Derepresses Silenced Tumor Suppressor Genes and Reverses the Drug-Resistant Phenotype in Cancer Cells

Phillip H. Abbosh[1], John S. Montgomery[1], Jason A. Starkey[2], Milos Novotny[2], Eleanor G. Zuhowski[3], Merrill J. Egorin[3], Annie P. Moseman[1], Adam Golas[1], Kate M. Brannon[1], Curtis Balch[1], Tim H.-M. Huang[4] and Kenneth P. Nephew[1]

[1]Medical Sciences, School of Medicine, [2]Department of Chemistry, Indiana University, Bloomington, IN 47405

[3]Department of Medicine, Division of Hematology-Oncology, University of Pittsburgh Cancer Institute, Pittsburgh, PA 15232

[4]Human Cancer Genetics Program, Department of Molecular Virology, Immunology, and Medical Genetics, Comprehensive Cancer Center, The Ohio State University, Columbus, OH, 43210, USA

Histone modifications and DNA methylation are epigenetic phenomena that play a critical role in many neoplastic processes, including silencing of tumor suppressor genes. One such histone modification, particularly at H3 and H4, is methylation at specific lysine (K) residues. While histone methylation of H3-K9 has been linked to DNA methylation and aberrant gene silencing in cancer cells, no such studies of H3-K27 have been reported. Here, we generated a stable cell line overexpressing a dominant-negative point mutant, H3-K27R, to examine the role of that specific lysine in ovarian cancer. Expression of this construct resulted in a 75% loss of methylation at H3K27, 63% global reduction of DNA methylation, and increased expression of tumor suppressor genes hMLH1, RASSF1A, and ARHI. One of the affected genes, *RASSF1*, was shown to be a direct target of H3-K27 methylation-mediated silencing. DNA-platinum adduct formation significantly increased in K27R cells after treatment with cisplatin, suggesting that a consequence of removal of H3-K27 methylation is increased access of the drug to target DNA sequences. The IC_{50} for cisplatin in K27R cells was 10 μM compared to 40 μM for CP70 cells, indicating that K27R cells were resensitized to the chemotherapeutic agent cisplatin. We also provide evidence that H3-K27 methylation is involved in DNA repair, as H3-K27 was methylated after treatment with cisplatin and loss of repair was observed in K27R cells. Our results demonstrate that overexpression of mutant histone sequences in mammalian cells represents a novel tool for studying epigenetic mechanisms and the Histone Code Hypothesis in human cancer. Such findings demonstrate the significance of H3-K27 methylation as a target for epigenetic-based cancer therapies.

The Ras-related GTPase, Rheb, inhibits MAPK signaling and promotes intracellular vesicle formation

Anderson, S.A. and Quilliam, L. A.

A role for the small GTPase, Rheb, has previously been suggested in H-Ras-induced transformation of NIH3T3 fibroblasts. Rheb is thought to function via an inhibitory binding with Raf. In addition, negative regulators of Rheb, including TSC1/2 and PTEN, are mutated in a number of cancers. To further delineate the role of Rheb1 and Rheb2 (RhebL1) in H-Ras signaling, these proteins were co-transfected with Ras-responsive promoters. Both Rheb isoforms were found to inhibit gene expression from a number of these constructs including the CyclinD1 promoter and an Elk-1-responsive promoter. This repression was tested to see if it was reversible by Rapamycin, an inhibitor of mTOR, a downstream target of Rheb that is critical for protein translation. The ability of Rheb to inhibit gene expression from the CyclinD1 promoter was blocked by Rapamycin, indicating the involvement of mTOR. This result is consistent with previous reports that regulation of CyclinD1 expression is Raf-independent and Rapamycin is an inhibitor of CyclinD1 signaling. Conversely, our research found the ability of Rheb to inhibit an Elk-1 responsive promoter is insensitive to Rapamycin. Expression of oncogenic H-Ras 61L, also failed to reverse this repression, indicating Rheb inhibits MAPK signaling downstream of H-Ras. Finally, expression of a constitutively active MEK mutant was sufficient to reverse the effect of Rheb on the Elk-1 responsive promoter suggesting an interaction with Raf (upstream of MEK but downstream of Ras) is potentially responsible for the inhibition. Previously, our lab has found regulation of another Ras-related GTPase, Rap1, varies depending on its subcellular location. To identify if Rheb functions in a similar manner, we used live cell imaging using green fluorescent protein chimeras to localize Rhebs. This work identified Rheb1 in a punctate, primarily perinuclear, pattern in LN229 brain tumor cells. A geranylgeranyl mutant of Rheb1, Rheb CLLL, was located primarily on small vesicular endomembranes of the cell, while a prenylation-defective mutant, Rheb1 SAAX, was found throughout the cell. Localization of the Rheb1 proteins was largely insensitive to Rapamycin. In contrast, both Rheb2 and a geranylgeranylated mutant, Rheb2 CHLL, were located on small vesicles that could be disrupted by Rapamycin treatment. This result suggests a role for mTOR complex 1 in formation of these small vesicles. Future work will focus on discovering a physiological role for these vesicles and analysis of mTOR signaling in this function.

MAPK-Dependent Regulation of CD1d-Mediated Antigen Presentation

Jennifer Bailey
Dr. Randy Brutkiewicz Laboratory
R2 Rm 359
Department of Microbiology and Immunology

The CD1d molecule is a glycoprotein that is similar in structure to MHC class I antigens, with a heavy chain bound to 2-microglobulin. Unlike MHC class I and MHC class II molecules that present peptides to the immune system, CD1d presents lipids. We have found in a virus infection model system that the mitogen-activated protein kinases (MAPK), p38 and extracellular signal-regulated protein kinases 1/2 (ERK1/2), reciprocally regulate CD1d-mediated antigen presentation. Activation of p38 is inhibitory (whereas activation of ERK1/2 promotes) antigen presentation by CD1d. In the current study, we have found that this reciprocal regulation of CD1d-mediated antigen presentation also occurs in breast cancer cells. CommaD-CD1d cells are a mouse mammary epithelial cell line transfected with a CD1d-expressing plasmid. These cells were also transfected with a plasmid overexpressing the oncogene H-Ras (CommaD-CD1dRas). Comma D-CD1dRas cells had higher levels of p38 activation, yet lower levels of ERK1/2 activation. Associated with this observation, H-Ras-transfected CommaD-CD1d cells were less able to activate NKT cells than those transfected with empty vector. Furthermore, treatment of CommaD-CD1d cells with a p38 inhibitor enhanced (but an ERK1/2 pathway inhibitor reduced) CD1d-mediated antigen presentation. To further support the negative control of CD1d-mediated antigen presentation by p38, CommaD-CD1d cells were transfected with a plasmid expressing a wildtype or dominant negative p38. NKT cell assays showed a significant increase in antigen presentation by CD1d when the DN form of p38 was expressed. This reciprocal regulation by p38 and ERK1/2 in our mouse breast cancer model indicates a novel means by which a tumor can evade an important component of the host's innate antitumor immune response, and demonstrates the overall importance of MAPK in the control of CD1d-mediated antigen presentation.

Biodisposition and metabolism of [^{18}F]fluorocholine (FCH) in cultured 9L glioma cells and subcutaneous 9L tumor model

Aditya Bansal, Shuyan Wang, Robert A. Harris, Toshiko Hara and Timothy R. DeGrado
Department of Radiology and Department of Biochemistry & Molecular Biology
Indiana University School of Medicine

Fluorine-18 labeled choline analogs are currently under investigation as PET imaging tracers for cancer. The objective of this work was to study the biodisposition and metabolism of [^{18}F]fluorocholine (FCH) in cultured 9L glioma cells and a subcutaneous 9L glioma rat tumor model. The cultured 9L glioma cells were incubated for 2 h in medium with FCH and [^{3}H]choline. Uptake and metabolism of tracers by cultured 9L glioma cells was compared. For subcutaneous 9L glioma rat tumor model, FCH and [^{14}C]choline were administered in isotonic saline by bolus injection through femoral vein. Blood was sampled from the carotid artery to compare the clearance of tracers from blood. At 5 and 20 min post-injection, the animals were euthanized, and uptake and metabolism in different organs were investigated by HPLC analysis. In this study, the uptake of both choline tracers was similar in cultured 9L glioma cells. Similar uptake was also seen in different tissues in the rat model, including tumor. Both FCH and choline were similarly phosphorylated and oxidized in cultured 9L glioma cells. In rat, similar metabolism of tracers in tissues was also seen. Rapid blood clearance of both tracers was seen in rat with variable secretion in urine with corresponding increase of betaine forms. These results showed similar biodisposition and metabolism of FCH as compared to choline. From this study we conclude that FCH closely mimics choline in tracer studies of uptake and metabolism.

Protein Phosphatase2A Regulates Stromal Cell- Derived Factor-1 Mediated Responses of CD34+ Cord Blood Cells.

Sunanda Basu, Barbara Graham-Evans and Hal E. Broxmeyer

Department of Microbiology and Immunology, Indiana University School of Medicine, Indianapolis; and the Walther Cancer Institute, Indianapolis, Indiana.

The chemokine stromal cell- derived factor-1 (SDF-1) and its receptor, CXCR4, play a major role in migration, retention, and development of hematopoietic progenitors in the bone marrow. During steady-state hematopoiesis, CXCR4/SDF-1 interaction restricts hematopoietic stem progenitor cells in marrow and disruption of the SDF-1/CXCR4 axis leads to their egress into circulation. However, biological responses to SDF-1 are rapidly attenuated by overlapping mechanisms that include peptide degradation by cell surface proteases, receptor uncoupling from heterotrimeric G-proteins and receptor endocytosis. It is known that phosphorylation of G-protein coupled receptors (GPCRs) by several protein kinases including serine-threonine kinase, protein kinase C (PKC), can result in receptor desensitization. This prompted us to investigate the role of serine-threonine phosphatase in the regulation of SDF-1 induced responses of CD34+ cord blood (CB) cells. To investigate the role of serine- threonine phosphatase we evaluated the effect of okadaic acid, a specific serine-threonine phosphatase inhibitor, on SDF-1 induced chemotaxis and adhesion of CD34+ cells from cord blood. Pre-incubation of CD34+ CB cells with okadaic acid (100-1000nM) significantly reduced SDF-1 directed chemotaxis and adhesion of CD34+ CB cells. This correlated with an increase in PKC phosphorylation. Although primary SDF-1 induced calcium flux in CD34+CB cells was unaffected by okadaic acid, if the cells were pretreated with SDF-1 and then stimulated with SDF-1, calcium flux was significantly less in CD34+CB cells pretreated with okadaic acid compared to untreated cells. Further, confocal microscopy was used to demonstrate the co-localization of serine threonine phosphatase and PKC. Using antibodies specific to protein phosphatase 2A catalytic subunit (PP2Ac) and various classes of PKC super family, it was observed in unstimulated CD34+ CB cells PP2Ac was mainly localized in nucleus and some phospho-PKC-delta was detected in the cytoplasm. However, upon SDF-1 stimulation, co-localization of PP2Ac and phospho-PKC-delta was observed on the membrane of CD34+CB cells. These results indicate that serine-threonine phosphatase regulate SDF-1 mediated responses in CD34+CB cells and may play an important role in the ability of hematopoietic stem progenitor cells to continually respond to SDF-1.

Human Papillomavirus Type 6 Reduces Retinoblastoma Family Member p130 Expression in Biopsies and Cell Culture.

Lisa Barrow-Laing[1,2,4], Darron Brown[1,3] and Ann Roman[1,2,4]. Department of Microbiology and Immunology[1] and Walther Oncology Center[2] and Department of Medicine[3], Indiana University School of Medicine, and Walther Cancer Institute[4], Indianapolis, IN.

Cervical cancer is the second most common cancer in women worldwide and is the leading cause of cancer-related deaths in women in developing countries. Human papillomavirus (HPV) is associated with over 99% of all cervical cancer. Certain genital HPV types, classified as high risk (HR), are associated with an increased risk of malignancy. Low risk (LR) HPV, such as HPV 6, is found in benign lesions. The E7 genes of HPV encode oncoproteins that bind to the retinoblastoma protein, pRb, and the pRb family members, p107 and p130. While E7 proteins of HR HPV can immortalize cells and induce proteasomal degradation of pRb, LR HPVs do not induce either activity. However, we have recently shown that both HR (HPV 16 E7) and LR E7 proteins (HPV 6 E7) can destabilize p130 when E7 is expressed in foreskin keratinocytes (Zhang et al., Proc. Natl. Acad. Sci. USA 130:437-442 (2006). This study was aimed at deciphering HPV 6 effects on p130 *in vivo*, and p130 localization in the presence of HPV 6 and HPV 16 E7 proteins. *In situ* hybridization (ISH) and immunohistochemistry (IHC) were performed on condylomata acuminate lesions caused by HPV 6. ISH, using an HPV 6 DNA probe, was performed to localize HPV 6 in tissues. Nuclear/cytosolic fractionation was also performed using HPV 6 E7 and HPV 16 E7 transduced human foreskin keratinocytes (HFKs). The protein fractions were then used for Western analysis and probed with antibodies against p130. A reduction of p130 expression level was seen *in vivo* by IHC in HPV 6 positive biopsies when compared to control foreskin tissue. Initial data generated by nuclear/cytosolic fractionation showed that E7 does not alter the sub-cellular localization of p130.

Identification of Post-translational Modifications of Estrogen Receptor alpha using Mass Spectrometry

Nicholas Berry[1], Randy Arnold[2,3], Milos Novotny[2,3], Kenneth Nephew[1]

[1]Indiana University School of Medicine [2]Indiana University Department of Chemistry
[3]National Center for Glycomics and Glycoproteome

Transcriptional activity and stability of estrogen receptor alpha (ER) are tightly regulated by several mechanisms, including post-translational modifications. For example, serine, threonine, and tyrosine residues can be targeted for phosphorylation, while lysine residues are substrates for acetylation and ubiquitination. The ER lysine residue(s) targeted for ubiquitination have not yet been identified. Mass spectrophotometry (MS) is a power technique capable of detecting increases in the molecular weight of proteins due to the addition of various post-translational modifications (PTMs). The purpose of this study was to utilize MS to span the ER protein sequence and reveal amino acid (AA) residues targeted for phosphorylation, acetylation, and ubiquitination. ER peptide (Panvera, Carlsbad, CA) was resolved by SDS-PAGE and visualized with Coomassie blue staining. A 66kDa band corresponding to ER was extracted, reduced with dithiothreitol and alkylated with iodoacetamide. ER was then digested overnight with trypsin. The resulting peptides were subjected to liquid chromatography followed by MS using an UltiMate-nano liquid chromatograph (LC Packings, Sunnyvale, CA) coupled to a Finnigan LCQ Deca XP MAX LC/MS (Thermo, Franklin, MA). Resulting sequences were analyzed using MASCOT, and fragments meeting the MASCOT 95% confidence score were considered as ER with a PTM. Several phosphorylated ER fragments were identified, as indicated by an 80Da increase in molecular mass. Peptides containing AAs S167, S282, S559, and T563 were phosphorylated. Of these AA residues, only S167 has been previously reported to be phosphorylated; the other phosphorylated AAs may be unique to this insect cell-expressed form of the receptor (the Panvera ERα peptide is expressed in SF9 cells). Acetylation at the N-terminus of ER, as indicated by a 42Da mass increase, was also observed. Previous reports have shown ER to be preferentially acetylated at lysines 302 and 303, suggesting N-terminal acetylation may also be related to SF9 cell expression. Using the MS approach, ubiquitinated lysine residues were not detected, perhaps due to the inherent instability of ubiquitinated proteins. In conclusion, this is the first report using MS to identify alternative phosphorylation and acetylation sites on ER. MS is a direct approach for visualizing PTMs and may be useful for identifying additional modifications on ER and other members of the nuclear receptor superfamily.

CXXC Finger Protein 1 (CFP1) Is Important for DNA Methyltransferase I (DNMT1) Stability

Jill Butler

Epigenetic regulation refers to changes in gene expression that occur without changing the nucleotide sequence. Cytosine methylation and histone tail modifications are two epigenetic modifications that influence gene expression. Elucidating epigenetic mechanisms of gene regulation is becoming increasingly important as deregulation of epigenetic processes is observed in many diseases, including cancer. Cells from a variety of cancers exhibit altered epigenetic modifications and inappropriate expression and activity of enzymes that establish and maintain these marks within the cell. These changes lead to altered gene expression, such as the silencing of tumor suppressor genes and improper expression of genes encoding signaling proteins (1,2). The *CXXC1* gene encodes CXXC finger protein 1 (CFP1), a transcriptional activator that specifically binds unmethylated CpG dinucleotides (3). The specific binding activity of CFP1 makes it unique in that most CpG binding proteins bind methylated CpG dinucleotides and facilitate heterochromatin formation. CFP1 has recently been identified as a member of the mammalian SET1 histone H3 lysine 4 methyltransferase complex (4). Disruption of *CXXC1* in mice results in an early embryonic lethal phenotype, therefore *CXXC1-/-* embryonic stem (ES) cells were isolated from *CXXC1-/-* blastocysts to further characterize the function of CFP1 (5). Embryonic stem cells lacking expression of CFP1 exhibit multiple epigenetic modification defects including altered histone modifications and reduced global cytosine methylation (6). DNA methyltransferase 1 (Dnmt1) is the major source of DNA methyltransferase activity in mammalian cells and is responsible for copying methylation patterns during DNA replication (7). Dnmt1 protein level and methyltransferase activity are decreased by ~50% in *CXXC1-/-* ES cells (6). Dnmt1 transcript level is not reduced, however Dnmt1 protein stability is decreased in *CXXC1-/-* ES cells. This finding prompted further investigation into the functional relationship between CFP1 and Dnmt1. Immunoprecipitation experiments revealed an interaction between CFP1 and Dnmt1 *in vivo*. Regulation of Dnmt1 protein level and activity by CFP1 is an exciting discovery in that it is the first example of reduced Dnmt1 protein without direct disruption of Dnmt1 gene function. It would be of interest to determine if CFP1 plays a role in stabilizing Dnmt1 in cancer cells overexpressing Dnmt1. The functional significance of this novel intersection of epigenetic regulatory proteins is the principle focus of ongoing experiments.

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The HMGA family member, p8 facilitates tumorigenesis through genomic instability and dysregulation of senescence.

K.M. Brannon, C.R. White, C. M. Million Passe, N.A. Bade, C. C. Quirk

The HMGA family member, p8, is a transcription factor required for tumor development in a variety of cell types. Our goal is to determine how p8 imparts its tumorigenic potential in the gonadotrope tumor-derived LβT2 cell line. Using a population of cells with mixed ploidy (84% ± 2.4 diploid, 13% ± 3.5 aneuploid); pools of stable cells were created in which p8 levels were manipulated. Control LβT2 cells (C-LβT2$^{dip/aneu}$) that stably express empty vector express p8 and maintain a predominantly aneuploid population (90 ± 1.5%) with 52 ± 12 chromosome pairs per cell (normal = 40). In culture, C-LβT2$^{dip/aneu}$ cells are resistant to stress-induced senescence and G_0/G_1 arrest. When injected subcutaneously into athymic nude mice, these C-LβT2$^{dip/aneu}$ cells quickly form tumors (25 ± 8 days post-injection) with 100% penetrance. In contrast, cells in which p8 has been stably knocked down using a full-length antisense construct, p8-KD-LβT2^{dip}, maintain a 100% near-diploid population of cells with 38 ± 9 chromosome pairs per cell. These diploid cells undergo senescence and appear to maintain cell cycle checkpoints, with a greater number of cells in G_0/G_1 when grown at high densities. In addition, compared to C-LβT2$^{dip/aneu}$ cells, p8-KD-LβT2^{dip} cells do not readily form tumors. Initial tumor formation was detected 119 days following injection of p8-KD-LβT2^{dip} cells. Even 300 days post-injection, tumor formation was observed in only 3 of 10 nude mice injected with these cells. Furthermore, p8 gene expression was similar in tumors formed from both cell types, suggesting that cells within the p8-KD-LβT2^{dip} pool in which p8 was not sufficiently knocked down were the cells with tumorigenic potential. To determine the potential importance of aneuploidy in the tumorigenic directive of p8 in LβT2 cells, we constructed a second set of stably-transfected cell lines in which we manipulated p8 levels using parent LβT2 cells that were aneuploid. This resulted in both control (C-LβT2^{aneu}) and knockdown (p8-KD-LβT2^{aneu}) cells that were also fully aneuploid. Both of these cell lines form tumors in nude mice at the same rate as C-LβT2$^{dip/aneu}$ cells. Our data supports the hypothesis that p8 facilitates tumorigenic events by allowing aberrant cells to avoid senescence and/or by inducing aneuploidy that drives oncogenesis.

The Role of CD26 in Human Umbilical Cord Blood $CD34^+$ Cell Engraftment of NOD/SCID Mice

Tim Campbell, Giao Hangoc and Hal E. Broxmeyer
Department of Microbiology & Immunology and the Walther Oncology Center, Indiana University School of Medicine, and the Walther Cancer Institute, Indianapolis, IN 46202

CD26 is a surface serine protease expressed on many different cell types, including human and mouse hematopoietic cells. CD26 cleaves the N-terminal dipeptide from some chemokines, including Stromal-Derived Factor 1 (SDF-1/CXCL12). SDF-1 plays an important role in hematopoietic cell homing and mobilization into and out of the bone marrow. The cleavage of SDF-1 by CD26 produces an inactive form which negatively affects human $CD34^+$ cord blood cell chemotaxis *in vitro*, and inhibiting the CD26 protease activity using the small peptide Diprotin A causes an enhancement of *in vitro* chemotaxis to SDF-1 (Christopherson II *et al* (2002). The Journal of Immunology 169: 7000-7008). In addition, this treatment causes an enhancement of *in vivo* homing, engraftment and competitive as well as non-competitive repopulation of murine hematopoietic stem cells in lethally irradiated congenic recipients (Christopherson II *et al* (2004). Science 305:1000-1003). The goal of our current studies was to determine what role CD26 plays in the *in vivo* engraftment of hematopoietic stem cells from human umbilical cord blood (hUCB). In order to answer this question, we used the sublethally irradiated NOD/SCID mouse model, which is one of the best *in vivo* tools available for studying human hematopoietic stem cell function. We isolated $CD34^+$ cells from hUCB and transplanted these cells into NOD/SCID animals after treating the $CD34^+$ cells with or without Diprotin A or a polyclonal goat anti-human CD26 antibody with isotype as control. In preliminary experiments, we showed that pretreatment of $CD34^+$ cells with either Diprotin A or a polyclonal goat anti-human CD26 antibody resulted in a greater percentage of animals with detectable engraftment compared to control as measured by flow cytometry and PCR (83% in pretreated group versus 25% in control group). In a further experiment, we found that pretreating purified $CD34^+$ human cord blood cells with Diprotin A caused a significant enhancement (> 3 fold) of engraftment in NOD/SCID mice at 6 weeks post-transplant as measured by the percentage of human $CD45^+$ cells in the mouse bone marrow (average chimerism of 6.5% in control group versus 23.9% in Diprotin A group). The relative proportion of human engrafted cells expressing four different blood cell markers (CD33, CD38, CD19 and CD34) did not significantly differ between the control and Diprotin A groups, suggesting that Diprotin A treatment does not significantly alter hematopoiesis towards one lineage at the expense of another lineage. We also investigated the nature of the $CD26^+$ population contained within the human $CD34^+$ cells by staining these cells with CD34, CD26 and CD38 antibodies and analyzing by flow cytometry. The $CD34^+CD38^{lo/-}$ population represents a more immature population of hematopoietic stem/progenitor cells. The percentage of $CD26^+$ cells within this population is 2-fold higher than the percentage of $CD26^+$ cells in the $CD34^+CD38^+$ population (12.1% compared to 5.7%, respectively, average of 4 experiments). This may suggest that immature stem/progenitor cells express CD26 preferentially. These results suggest that inhibiting CD26 on immature subsets of CD34+ cells enhances the engrafting capability of these cells in sublethally irradiated NOD/SCID mice. Clearly, further studies are needed to determine the full extent of these effects, information that may be of use for enhancement of the clinical engrafting capability of limiting numbers of stem cells.

Structure function analysis of hematopoietic specific Src family kinases Lyn, Hck and Fgr reveal a critical role for Lyn's unique domain in regulating growth of myeloid cells that is distinct from the role of Hck and Fgr's unique domain

Jinbiao Chen, Sasidhar Vemula, and Reuben Kapur*. Department of Pediatrics, Herman B Wells Center for Pediatric Research, Indiana University School of Medicine, Indianapolis, IN, USA.

A flurry of recent publications have implicated hematopoietic specific Src family kinases (SFKs) in contributing to the pathogenesis of lymphoid and myeloid leukemia, including in regulating the survival of drug resistant, BCR-ABL positive leukemic cells. Furthermore, in preclinical models of leukemogenesis, deficiency of Hck, Fgr and Lyn has been shown to significantly modulate the development of B lymphoblastic leukemia. In spite of these advances, little is known about the mechanism(s) by which these kinases regulate the growth of myeloid cells. A better understanding of the relative contribution of specific domains of hematopoietic specific Src family kinases in regulating the growth of myeloid cells would allow for the design of drugs that could specifically target the function of a specific Src family member. Src family tyrosine kinases share significant structural and amino acid sequence homology, particularly in the catalytic domain and in both the SH2 and SH3 domains of the regulatory region. Based on the high degree of structural homology, it is generally believed that various Src family kinases play a redundant role in regulating growth and actin based functions. Although, there are no apparent differences in the known effector domains of Src family kinases, significant differences exist in the amino terminus unique region of Hck, Fgr and Lyn. Here, we show that the deficiency of Hck or Fgr or both in myeloid cells results in reduced cytokine (stem cell factor [SCF] & IL-3) induced proliferation. In contrast, deficiency of Lyn SFK results in enhanced growth and survival of myeloid cells. SCF induced hypersensitivity due to Lyn deficiency is observed in spite of the presence of Hck and Fgr in these cells, suggesting that Lyn SFK functions with specificity in negatively regulating cytokine signaling in myeloid cells. To determine whether SCF induced hypersensitivity due to Lyn deficiency is contributed via its N-terminal unique domain, we cloned and retrovirally expressed a mutant version of Lyn that lacks its unique domain. As expected, reconstituting *Lyn-/-* myeloid cells with a cDNA encoding the wildtype version of Lyn completely restored cytokine induced hypersensitivity to wildtype levels. In contrast, expression of a Lyn mutant lacking its N-terminal unique domain, but consistent of all other domains, including the myristolation and the palmitoylation acetylation sites behaved in a fashion similar to Lyn deficient myeloid cells. Furthermore, neither overexpression of Hck, or Fgr in *Lyn-/-* cells affected cytokine induced hypersensitivity. To further characterize the unique nature of Lyn's N-terminal (unique) domain in cytokine induced hypersensitivity, we cloned and expressed several chimeric proteins in which the unique domain of Lyn was replaced with the unique domain of Hck or Fgr. When expressed in *Lyn-/-* cells, these chimeric proteins were unable to correct cytokine induced hypersensitivity due to Lyn deficiency. In contrast, swapping Hck's or Fgr's unique domain with Lyn's unique domain completely rescued cytokine induced hypersensitivity in *Lyn-/-* cells. These results suggest that Lyn's unique domain plays a critical role in negatively regulating cytokine induced growth in myeloid cells. Although Lyn contains a palmitoylation and an acetylation site, the specific role of these sites in membrane targeting and in cytokine-induced growth are not known. To assess the role of these sites, we generated three additional mutants of Lyn and expressed them in *Lyn-/-* cells. Our results suggest that the mutants of Lyn lacking both the acetylation and the palmitoylation sites demonstrate cytokine induced hypersensitivity similar to that seen in *Lyn-/-* cells. In contrast, mutating just the palmitoylation site alone in Lyn had no affect on cytokine induced hyperproliferation, although mutating the acetylation site led to cytokine induced hypersensitivity reminiscent of Lyn deficiency. Taken together, these results suggest that in addition to the lipid modification sites of Lyn, Lyn's unique domain also contributes to cytokine signaling, which is distinct from the role of Hck or Fgr's unique domain.

HUMAN PAPILLOMAVIRUS 16 E6/E7 CAUSES AN ANGIOGENIC SWITCH IN KERATINOCYTES WHICH IS SUFFICIENT TO ALTER ENDOTHELIAL CELL BEHAVIOR.

[1] W. Chen, [2]L. Fang, [2]L. Mead, [2]H. White, [3]A. Klingelhutz, [2]D. Ingram, and [1]A. Roman.[1]Department of Microbiology and Immunology and the Walther Oncology Center, Indiana University School of Medicine and the Walther Cancer Institute
[2]Department of Pediatrics, Herman B. Wells Center for Pediatric Research, Indiana University School of Medicine, Indianapolis, IN
[3]Department of Microbiology, University of Iowa, Iowa City, IA

One of the requirements for tumor growth is the ability to recruit a blood supply, a process known as angiogenesis. Analysis of cervical biopsies and mouse transgenic models indicates that the process of angiogenesis begins early in the progression of cervical disease from mild to severe dysplasia and on to invasive cancer. Because expression of angiogenic factors is regulated by tumor suppressors, we hypothesized that HPV gene expression could contribute to the angiogenic response since E6 and E7 target p53 and pRb for degradation. Consistent with this, we have previously reported that expression of HPV 16 E6/E7 transduced into primary foreskin keratinocytes (HFKs) decreases expression of two angiogenic inhibitors maspin and thrombospondin and increasing expression of two angiogenic inducers VEGF and IL-8 (Toussaint-Smith et al., 2004). Here we report on in vitro and in vivo experiments that were undertaken to test whether these changes in keratinocyte phenotype were sufficient to alter endothelial cell behavior. In the in vitro assays, conditioned media from HPV 16E6/E7 expressing HFKs or control HFKs were incubated with human microvascular endothelial cells (HMVECs) and the ability of the media to stimulate proliferation of HMVECs was determined. Alternatively, the conditioned media was placed in the lower chamber and HMVECs in the upper chamber of a transwell plate and the number of HMVECs migrating through the membrane was determined. The conditioned media from HPV 16E6/E7 expressing HFKs stimulated the HMVECs to both proliferate and migrate, two endothelial cell functions linked to neoangiogenesis. The matrigel plug assay was used in vivo. Introduction of conditioned media from HFKs expressing HPV 16E6/E7 into the matrigel and subsequent injection of the matrigel into strain 129 mice resulted in a clear angiogenic response documented both by the visual evidence of blood in the matrigel plug and the quantitation of hemoglobin. Further experiments, conducted with cervical cells transfected with the intact HPV 16 genome also stimulated proliferation and migration in vitro and an angiogenic response in vivo above the background seen with control cervical cells. These data support the hypothesis that HPV gene products contribute not only to the uncontrolled keratinocyte growth seen following HPV infection but also to the angiogenic response needed for tumor formation.

Ape-1/ref-1 Mediated Redox Regulation of Retinoic Acid Receptor Function in Myeloid Leukemia Cell Differentiation and Apoptosis

Edwin Colvin, Meihua Luo, Ashley Chastain, April Reed, Mark R. Kelley, and Kent A. Robertson. Department of Pediatrics, Indiana University, Riley Hospital for Children, Wells Center for Pediatric Research, Indianapolis, IN 46202

Ape1/ref-1is a multifunctional base excision DNA repair protein that is involved in the repair of abasic sites in DNA. However, it also has a distinct role in the redox regulation of a variety of cellular proteins, such as Fos, Jun, p53, NFkB, PAX, HIF-1a, HLF, and others. Ape-1/ref-1 maintains these proteins in a reduced state thereby facilitating their DNA binding and transcriptional activation capability. HL-60 cells are known to respond to retinoic acid (RA) with terminal granulocytic differentiation and apoptosis, which is mediated through the RA receptors. Previous experiments suggested that Ape1/ref-1 expression is related to apoptosis. To further define this relationship, we used retroviral gene transduction to over-express HA-tagged Ape1/ref-1 in HL-60 myeloid leukemia cells. We observed that the RA-induced growth inhibition of HL-60 cells over-expressing Ape1/ref-1 was significantly enhanced compared to wild type HL-60 cells. To determine if the growth inhibition was related to enhanced programmed cell death and differentiation, we treated Ape1/ref-1 transduced and vector-only (LXSN) transduced HL-60 cells with RA and evaluated the expression of Ape1/ref-1 and the development of apoptosis and markers of differentiation. Results: 1) RA induced expression of the retroviral Ape1/ref-1 construct as determined by Western blot resulting in a higher (ie retroviral + endogenous Ape1/ref-1) overall expression of Ape1/ref-1 compared to control cells; 2) analysis of RA-treated cells for apoptosis by propidium iodide, TUNEL, and Annexin V staining as well as morphology, unexpectedly demonstrated enhanced programmed cell death in cells expressing the transduced Ape1/ref-1; 3) Ape-1 over-expression enhanced the retinoid differentiation response by morphology and expression of CD11b. Additional mobility shift experiments demonstrated the redox dependence of retinoic acid receptor binding to retinoid response elements mediated by Ape-1/ref-1. Preliminary results of co-transfection experiments support a role for Ape-1/ref-1 in stimulating RA activated RAR mediated transcription of a reporter gene. In conclusion, our data supports the contention that Ape1/ref-1 expression may be important for mediating RA-induced myeloid differentiation and programmed cell death.

Examination of the potential pathological role played by periostin within the tumor microenvironment.

Simon J Conway, Haiyan Wang, Hongming Zhou, Wei Li, Paige Snider, Jian Wang, Rhonda Rogers.
Herman B Wells Center for Pediatric Research, Riley Hospital for Children, Indiana University School of Medicine, 1044 W. Walnut, Indianapolis, IN 46202 (siconway@iupui.edu)

Periostin is a secreted TGF -inducible ECM adhesion protein that was originally isolated as *osteoblast-specific factor-2*. The gene has structural similarity to *fasciclin-I*, a *Drosophila* protein involved in guidance of migrating cells, and there are both membrane-associated and secreted forms. The *fasciclin* extracellular domain is repeated four times in *periostin* and is evolutionary conserved from man to bacteria. Periostin can support osetoblast attachment and spreading, and may be a ligand for $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins and promote integrin-dependent cell adhesion and enhance cell motility. It is expressed throughout the body and is highly expressed in both normal embryonic/adult tissues and several pathological conditions. Multiple reports have demonstrated elevated serum levels in tumor samples from neuroblastoma, head/neck carcinoma, epithelial ovarian cancer, lung cancer and in breast cancer patients with bone metastases that had undergone epithelial-mesenchymal transformation and migrated. Significantly, *periostin* potently promotes metastatic growth of colon cancer by augmenting cell survival via the Akt/PKB pathway[1]. Furthermore, *periostin* has structural and sequence homology with another TGF -inducible gene, *igH3*, that is upregulated in colorectal cancers and liver metastasis but downregulated in primary lung carcinomas. Alterations in *igH3* expression levels have been linked to progression of retinoblastoma, colorectal, pancreatic, ovarian and oral squamous cell carcinomas. Significantly, exposure of colorectal cancer cells to anti-periostin antibodies activates apoptosis and potentiates the effects of 5-fluorouracil chemotherapy[2]. Whilst *igH3* over-expression inhibits cell attachment *in vitro* and a decrease in the ability of transfected CHO cells to form tumors in nude mice[3]. Although these data support a role for these two *fasciclin*-containing targets in the differential modulation of cell adhesion and tumor formation, further basic research is required before targeting these proteins as potential therapeutic options.

In order to clarify the role of the *periostin* gene in the pathogenesis of these various human cancers, we used human tissue-array profiling. We have also begun to characterize both *periostin* and *igH3* mRNA and protein expression in mouse tumors and several *in vitro* cancer cell lines. Specifically we are interested in determining whether it is produced from the tumor itself or the stromal cells adjacent to the cancer cells. Finally, in order to definitively test the requirement of *periostin* and *igH3* within the tumor microenvironment, we generated both *peri*^{lacZ}[4] and *igH3*^{lacZ} null mice mutants. As both are viable, this will enable future testing of the function of these two *fasciclin*-containing targets *in vivo*, and allow us to clarify the potential tumor suppressor function of igH3 and metastasis-promoting effects of periostin.

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THE ROLE OF KUPFFER CELLS IN HEPATOCARCINOGENESIS

S.M. Corthals, A.M. Keeney, M.T. Hufford, and L.M. Kamendulis. Department of Pharmacology and Toxicology, Indiana University School of Medicine, Indianapolis, IN

The induction of chemically induced hepatic neoplasia is a multi-step process involving DNA damage and cell proliferation. Historically, research has focused on the hepatocyte as the target cell of chemical carcinogens, however, recent studies have suggested a role for nonparenchymal cells, specifically Kupffer cells, as mediators of cell proliferation by tumor promoters. Through their activation, Kupffer cells secrete bioactive products that may participate in hepatocarcinogenesis through inducing oxidative stress and damage, altering signaling cascades, and/or increasing cell proliferation. Our group has further examined the role of the Kupffer cells on hepatocellular growth in both in vitro and in vivo models. Wyeth 14,643, a nongenotoxic hepatic tumor promoter, activated primary cultured Kupffer cells by inducing TNFα release in a concentration- and time-dependent manner. Wyeth also increased DNA synthesis in standard (non-purified) hepatocyte cultures in a concentration-dependent manner, however, in pure cultures of hepatocytes (lacking Kupffer cells) no increase in DNA synthesis was observed. p38 MAP kinase has been shown to be involved in signaling pathways that lead to TNFα release in macrophages, and plays an important role in cellular responses to external stress signals. Wyeth-induced phosphorylation of p38 was seen in standard hepatocyte cultures, but was not induced by Wyeth in pure hepatocyte cultures, as shown by western blot analysis. In vivo, male B6C3F1 mice treated with LPS resulted in an increase in the number of Kupffer cells and DNA synthesis in hepatocytes. Conversely, depletion of Kupffer cells via liposomal delivery of clodronate reduced the number of Kupffer cells by approximately 80% and also reduced both basal and LPS-induced DNA synthesis in the liver. These data demonstrate that activation of Kupffer cells stimulate hepatocellular growth while depletion of Kupffer cells inhibit hepatocellular growth and provide further support for the involvement of the Kupffer cell in hepatocarcinogenesis.

Gender Differences in Depression in a Cancer Population Assessed with the Patient Health Questionnaire-9

Joshua Dyer, Caroline Carney-Doebbeling, Lori Losee

Background: It is commonly reported that women are twice as likely as men to meet criteria for major depression in the general population. However, little research has focused on gender differences in the presentation of depressive symptoms in cancer patients. Objective: To determine whether the presentation of depressive symptoms differs between men and women presenting for cancer treatment. Methods: A waiting room sample of 282 (men n=191; women n=91) patients with colorectal and lung cancer in a tertiary care, multi-disciplinary cancer center completed a clinical triage questionnaire containing the Patient Health Questionnaire-9 (PHQ-9) and the Memorial Distress Thermometer. Major depression was coded when an individual endorsed either anhedonia and/or "feeling down, depressed, or hopeless," five of nine total depression symptoms, and distress ("If you checked any problems on this questionnaire, how difficult have these problems made it for you to do your work, take care of things at home, or get along with other people?"), thus closely mirroring DSM IV criteria.
Results: Overall, 8% (n=23) of all subjects met criteria for major depression and 7% (n=19) for subsyndromal depression. Women were more likely than men to endorse anhedonia (p=.02), feelings of depression (p=.02), sleep difficulty (p=.02), eating changes (p<.001), and distress (p=.03). However, women were not significantly more likely than men to meet criteria for major depression (chi-square=.82, *ns*) or subsyndromal depression (chi-square=.74, *ns*). 114 patients scored at or above the cut-off of 5 on the Memorial Distress Thermometer. Women were significantly more distressed than men (mean score=4.4 (SD=3.2) vs. 3.3 (SD=3.0);$t(264)=2.7$, p=.008). Distress was significantly correlated with major depression (r^2=.36, p<.001).
Conclusions: Major depression and subsyndromal depression are common in cancer patients. While women are more likely to endorse several symptoms of depression and experience more social distress, gender does not appear to be a risk factor for major depression in cancer patients. Social distress is moderately associated with the presence of major depression.

***Nf1* plays an important role in modulating mesenchymal stem cell migration and adhesion**.

Selina A. Estwick, Xiaohua Wu, and Fengchun Yang.
Herman B. Wells Center for Pediatric Research, Department of Pediatrics. Indiana University School of Medicine. Indianapolis, IN 46202

NF1 is a dominant genetic disorder which affects 1 in 3500 individuals. NF1 occurs as a consequence of mutation of *NF1* tumor suppressor gene. *NF1* encodes a GTPase-activating protein called neurofibromin that negatively regulates Ras signaling. Neurofibromas, a hallmark of NF1, are composed of fibroblasts, Schwann cells, mast cells and endothelial cells. 80% of the dry weight of neurofibromas is collagen, which is the major source of fibroblasts. Given that fibroblasts are the one type of progenies of mesenchymal stem/progenitor cells (MS/Ps), we sought to understand the role of NF1 in modulating the biological functions of MS/Ps utilizing a *Nf1* heterozygous (+/-) murine model. MS/Ps were generated from bone marrow mononuclear cells collected from wildtype (WT) and *Nf1+/-* mice. Uniformed population of MS/Ps was phenotypically analyzed by flow cytometeric analysis, and later was utilized for multiple biological functional assays, including migration, adhesion, and cell survival. While there is an induction of migration in WT MS/Ps with 5% fetal calf serum, a significantly higher migration was observed in *Nf1+/-* MS/Ps by a transwell assay in the presence of 5% fetal calf serum. This result was confirmed by a wound healing assay. Adhesion assay shows that a significantly higher number of *Nf1+/-* MS/Ps was observed adhering to 5 1 integrin as compared to WT cells. In addition, *Nf1+/-* cells are larger and contain a stronger f-actin expression as compared to WT cells. Importantly, *Nf1+/-* MS/Ps have increased cell survival because of their ability to resist anoikis by overexpressing receptors of integrins such as CD49e. Collectively, haploinsufficiency of *Nf1* results in gain in migration, adhesion, and survival of MS/Ps. Our study implies that *Nf1* plays an important role in regulating MS/Ps functions. Further investigation to understand the biochemical pathway(s) mediating the biological functions is ongoing.

Chemotherapeutic selectivity conferred by selenium: a role for p53-dependent DNA repair

Joshua L. Fischer and Martin L. Smith

[1]*Department of Microbiology and Immunology, Walther Oncology Center, Indiana University Cancer Center, and Walther Cancer Institute, Indiana University School of Medicine, Indianapolis, IN 46202 USA*

Selenium in various chemical forms has been the subject of cancer chemoprevention trials, and currently is being investigated as an adjuvant in combination with DNA-damaging chemotherapeutics. Recent xenograft studies in mice have demonstrated a capacity for selenomethionine to alleviate the toxic effect of chemotherapy drugs and increase the maximum tolerated dose, without protecting cancer cells (S. Cao et al, Clin Cancer Res. 2004). This observation and our previous work led us to hypothesize that a DNA repair response may be associated with the selenium protection of normal tissues. The tumor suppressor p53 is mutated in the vast majority of cancers, but is by definition wildtype in nontarget tissues such as bone marrow and gut epithelium, tissues that are often dose-limiting due to DNA damage. Furthermore, p53 is known to govern nucleotide excision DNA repair. Thus, p53 is a potential molecular determinant for selenium-induced chemotherapeutic selectivity. To test the hypothesis we assayed DNA repair and cell survival in an isogenic system using wildtype and p53 -/- primary mouse embryo fibroblasts. The wildtype cells had elevated DNA repair and were protected from DNA damage when selenomethionine treatment preceded administration of exogenous DNA damage. DNA repair in the p53 -/- cells was not affected and the cells remained sensitive to DNA damage when pretreated with selenomethionine. To provide a model for antitumor selectivity, we repeated DNA repair and cell survival experiments using primary rat gut epithelial cells, and primary mouse bone marrow cells as examples of nontarget tissues relevant to the chemotherapeutic system. In addition, we used two human cancer lines that were used in the recent xenograft studies, both squamous cell carcinoma of the head and neck that have known defects in p53. The bone marrow and gut epithelial cells treated with selenomethionine exhibited an elevated DNA repair response and were protected from DNA damage while the cancer cells did not have altered DNA repair and remained sensitive to DNA damage. These studies provide a potential molecular basis for chemotherapeutic selectivity conferred by selenium.

Amphireglulin-EGFR signaling regulates PTHrP gene expression in breast cancer cells

Jennifer L. Gilmore, Jeff Scott, Sarah Pitfield, David J. Riese II and John Foley
Indiana University, Bloomington, Indiana 47405 Purdue University, West Lafayette, IN 47907

Nearly 70% of cases of invasive breast cancer will result in metastasis to the bone and the vast majority of the 45,000 breast cancer deaths each year are associated with the severe pain and disability that osteolytic bone metastasis produces. An autocrine/paracrine factor produced by breast cancer cells called parathyroid hormone-related protein (PTHrP) is speculated to play a major role in permitting breast cancer cells to grow into the bone microenvironment by stimulating the bone resorption axis. It has been previously shown that EGFR signaling induces the production of PTHrP in several primary and transformed epithelial cell types. Therefore, we investigated the relationship between EGFR and PTHrP gene expression in human breast cancer cells.

Among a panel of 8 breast epithelial and cancer cell lines, the bone seeking EGFR positive lines exhibited higher levels of PTHrP transcript expression. Using QRTPCR, amphiregulin mRNA levels in all lines were approximately 2 orders of magnitude higher than those of TGF or HB-EGF. Amphiregulin mRNA levels were also elevated in the bone seeking lines. Among the EGFR tyrosine residues that mediate interactions with 2nd messenger pathways, only Tyr992 was phosphorylated under basal conditions the bone seeking lines. Treatment of these lines with the EGFR inhibitor PD153035 (1 M) and amphiregulin-neutralizing antibodies reduced PTHrP mRNA levels by 60 to 70%. In addition, 1 M of the EGFR tyrosine kinase inhibitor PD153035 blunted TGF -stimulation of PTHrP transcription by 50%. Retroviral mediated transduction was used to express the EGFR in the MCF7 line that expresses minimal levels of EGFR and PTHrP. Stable EGFR expression in the MCF7 line does not increase basal PTHrP production; however, treatment of this cell line with EGF or amphiregulin stimulates PTHrP expression 3-fold. Moreover, inhibition of PKC or Raf blunt EGFR mediated activation of PTHrP gene expression. Taken together, it appears that autocrine production of amphiregulin stimulates PTHrP gene expression through second messenger pathways downstream of EGFR Tyr992 (PLC /PKC/Raf/Ets) and this may contribute to expression of the peptide in breast cancer cells within the bone microenvironment. Furthermore, Tyr 992 maybe a marker for amphiregulin-induced EGFR signaling in breast cancer cells.

A High-Throughput, High-Resolution Strategy for the Rapid Structural Elucidation of Site-Selective DNA Binding Agents

Kristie D. Goodwin,[§] Mark A. Lewis,[‡] Millie M. Georgiadis*[,§] and Eric C. Long*[,‡]

Departments of Biochemistry & Molecular Biology, Indiana University School of Medicine[§] *and Chemistry & Chemical Biology, Purdue School of Science,*[‡] *Indiana University-Purdue University Indianapolis (IUPUI), Indianapolis, Indiana 46202*

E-mail: mgeorgia@iupui.edu, long@chem.iupui.edu

A general strategy for the rapid structural analysis of DNA binding ligands is described. By combining a high-throughput fluorescent intercalator displacement (HT-FID) assay and a high-resolution (HR) host-guest crystallographic technique, a strategy was produced that is capable of determining detailed structural information pertaining to DNA-ligand interactions within ~ 3 days [1]. This "HT-HR" strategy can quickly reveal the binding site preferences for even an unstudied DNA-interacting ligand and, based on these site-selectivities, oligonucleotides can be designed and the host-guest crystallographic method used to generate diffraction quality crystals, at times, *overnight*. Using the HT-HR strategy, we have been examining the DNA interactions of: (1) **RT-29**, a new benzimidazole-diamidine compound that displays anti-trypanosomal activity; (2) the minor groove binding natural product netropsin [2]; and (3) the clinically employed anticancer agent bleomycin, which induces C4´-H abstraction and single- and double-strand DNA scission. Our analyses suggest that the HT-HR strategy may be used to expedite the screening of novel DNA binding and damaging agents, including libraries of potential DNA-interacting compounds.

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Studies of genomic instability during breast tumorigenesis

Nazmul Huda[1], Hiromi Tanaka[1], Brittney-Shea Herbert[1], Robert L. Bacallao[2] and David Gilley[1]

[1]Department of Medical and Molecular Genetics, Indiana University School of Medicine, Indianapolis, Indiana 46202, [2]Department of Nephrology, Indiana University School of Medicine, Indianapolis, Indiana 46202

Telomeres are specialized DNA/protein structures that act as protective caps that function to prevent chromosome end-to-end fusions. The prevention of chromosome end-to-end fusions by functional telomere caps is a critical component in the maintenance of genomic integrity. Loss of telomere capping causes chromosome ends to fuse, leading to subsequent breakage-fusion-bridge cycles resulting in genomic instability.

Dysfunction at the telomere is likely a key driving force behind the genomic instability observed in early cancer lesions and has been implicated as an important factor in age-related diseases. Since genomic instability is one of the earliest neoplastic changes known to occur in tumorigenesis, determining its cause(s) is critical for understanding the etiology of cancer. Several recent reports support the theory that defects in telomere maintenance initiate genomic instability eventually resulting in the development of breast cancer and other cancers. However, the extent of telomere dysfunction in human cancers has not been directly determined due to limitations in detecting telomere dysfunction within tissue. This research focuses on the detection and analysis of telomere dysfunction in human breast tumor tissue, a critical problem in elucidating mechanisms responsible for the origin of genomic stability. Such understandings are expected to facilitate translational applications for early detection and therapeutic targeting strategies for cancer and age-related disorders.
We have developed a new method to detect and analyze telomere fusion from breast tumor tissue to directly test the telomere dysfunction hypothesis. Our preliminary studies suggest that: 1) breast tumors, but not normal tissue, contain telomere fusions; and 2) telomere fusion junctions contain not only telomeric repeats but also relatively short regions of non-telomeric, previously identified fragile DNA repetitive elements. As an outcome of these studies, it is expected that we will determine the extent and stage of telomere dysfunction in human breast cancer. Therefore, the combined positive impact as the result of these studies on both fields of telomere and cancer biology as a marker for early breast cancer detection is expected to be highly significant.

Differential effect of glycosphingolipid biosynthesis inhibitors on CD1d-mediated antigen presentation

Masood A. Khan, Venkataraman Sriram, G. J. Renukaradhya and Randy R. Brutkiewicz
Department of Microbiology and Immunology,
Indiana Univ. School of Medicine and the Walther Oncology Center,
Indianapolis, IN 46202, and the Walther Cancer Institute, Indianapolis, IN 46208

CD1d molecules present glyco- and phospholipids antigens to a unique subset of T lymphocytes called NKT cells. Prototypical mouse NKT cells, often referred as invariant NKT (iNKT) cells, express a semi-invariant T cell receptor (TCR), with an α-chain composed of Vα14-Jα18 paired with a β-chain of limited diversity (e.g., Vβ8.2, Vβ7, or Vβ2). The aim of the present study was to investigate the effect of the glycosphingolipid inhibitors fumonisin B1 (FB1), PPMP, and N-butylgalactonojirimycin (NB-DGJ) on CD1d-mediated antigen presentation to NKT cells. Treatment of antigen presenting cells (APCs) with PPMP (1.25-20 μM) for 24 hours inhibited antigen presentation by CD1d in a dose-dependant manner. Unlike PPMP, the other inhibitors of glycosphingolipid biosynthesis that were used, FB1 (1.5-25 μM) and NB-DGJ (9-150 μM), did not inhibit CD1d-mediated antigen presentation. In a similar manner as PPMP, ceramide treatment (6.25-100 μM) also inhibited antigen presentation by CD1d. PPMP, ceramide, FB1, or NB-DGJ did not significantly alter cell surface levels of CD1d on the APCs. Treatment of APCs with PPMP or ceramide resulted in the activation of the mitogen activated protein kinase (MAPK) p38, whereas FB1 or NB-DGJ did not. Moreover, the p38 MAPK inhibitor, SB203580 (but not the ERK1/2 MAPK pathway inhibitor, U0126) rescued PPMP-induced inhibition of CD1d-mediated antigen presentation. Confocal microscopic analysis revealed diminished co-localization of CD1d and LAMP-1 in APCs treated with PPMP or ceramide, whereas APCs treated with FB1 or NB-DGJ showed CD1d/LAMP-1 co-localization comparable to vehicle treated cells. Therefore, these data suggest that PPMP and ceramide impair CD1d-mediated antigen presentation by altering the trafficking of CD1d molecules in a p38 MAPK-dependent manner.

TGF-β1 Regulates NKG2D Promoted Effector Differentiation by Induction of CD30 and Suppression of CD137 in Umbilical Cord Blood CD8$^+$ T Cells.

Young-June Kim, Hal E. Broxmeyer Department of Microbiology and Immunology, The Walther Oncology Center, Indiana University School of Medicine, and the Walther Cancer Institute, Indianapolis, IN, USA

CD8$^+$ T cells are crucial for host defense against pathogens and tumors. Upon, antigen recognition, CD8$^+$ T cells proliferate and acquire effector functions. Effector CD8$^+$ T cells require distinct costimulatory receptors at different differentiation stages that are involved in cell expansion, cytokine secretion and cytolytic activity. NKG2D is an activating costimulatory receptor of CD8$^+$ T cells important for promoting effector responses of these cells to autoimmunity, virus infection and tumor immunity. CD8$^+$ T cell responses are also counterbalanced by various suppressive immune factors such as TGF-β1 or CD4$^+$CD25$^+$ regulatory T cells which plays a central role in maintenance of peripheral immune tolerance. We investigated whether NKG2D stimulation leads to induction of additional costimulatory receptors such as CD137 and CD30, which are important for the fate of effector functions and whether these NKG2D-mediated effects are affected by TGF-β1, using exclusively naïve CD8$^+$ T cells isolated from human umbilical cord blood. Following stimulation of NKG2D on naïve CD8$^+$ T cells with anti-NKG2D in the presence of anti-CD3, we examined induction profiles of CD137 and CD30, levels of granzyme B production, and cytotoxic activities against C1R lymphoblast tumor cell lines expressing MHC class I-related chain (MIC), a ligand for NKG2D. Even though naïve CD8$^+$ T cells from umbilical cord blood are hyporesponsive to anti-CD3, costimulation of NKG2D readily activated CD8$^+$ T cells and induced CD137 expression on cell surface. As a result of the NKG2D receptor ligation, NKG2D rapidly disappeared from the cell surface. These resulting CD8$^+$ T cells, expressing CD137 but lacking NKG2D on the cell surface, remained poor in cytotoxicity Costimulation of CD137 with CD3 restored NKG2D to the surface of CD8$^+$ T cells while further increasing CD137 expression by a positive feedback response. These NKG2D$^+$CD137$^+$CD8$^+$ T cells generated by CD137 stimulation were potent in cytotoxicity and produced high levels of granzyme B. NKG2D-induced CD137 expression was greatly compromised by TGF-β1. Unlike CD137, CD30, similar in molecular structure and belonging to the same TNF receptor family, was not induced by NKG2D stimulation. However, TGF-β1, which suppressed CD137 expression, appeared to be essential for NKG2D to induce CD30. TGF-β1 alone did not induce CD30 expression, suggesting TGF-β1 has a regulatory role for NKG2D in CD30 induction. Moreover, both CD30 and CD137 stimulation lowered effector functions compared to CD137 alone, suggesting that CD30 plays a negative role in CD137 costimulation. Our results suggest that a dichotomy between differential CD137 and CD30 induction on CD8$^+$ T cells after NKG2D stimulation may be important for the ultimate fate of the effector functions. TGF-β1 may be involved in regulating NKG2D-mediated effects on effector CD8$^+$ T cell development by induction of CD30 and reciprocal suppression of CD137 expression.

Fluorescent Intercalator Displacement Analyses of the DNA Binding Site-Selectivities of Peptide-Derived Anti-Cancer Natural Products: Netropsin, Actinomycin, and Bleomycin

Mark A. Lewis and Eric C. Long*

Department of Chemistry & Chemical Biology, Purdue School of Science, Indiana University-Purdue University Indianapolis (IUPUI), Indianapolis, Indiana 46202

E-mail: long@chem.iupui.edu

Elucidation of the binding site-selectivities of DNA-targeted anti-cancer agents is fundamental to a full understanding of their mechanism(s) of action. Unfortunately, traditional methods such as footprinting are slow and often cannot be carried out in a comprehensive fashion with regards to an evaluation of all possible DNA binding sites. The response of the high-throughput fluorescent intercalator displacement (HT-FID) assay reported recently by Boger *et al.* [1] to peptide-based, anti-cancer DNA binding intercalators and metal complexes was examined through the study of actinomycin and Co(III)•bleomycin-B_2 [2]. This assay permits the simultaneous evaluation of drug binding to all possible 4-, 5-, or other length base pair DNA binding sites in a 96-well plate format resulting in a rapid determination of binding site preferences. Along with a validation of netropsin that illustrated the good laboratory-to-laboratory reproducibility of the assay, our examination of actinomycin revealed results for a four base pair cassette library of DNA hairpins that paralleled the known DNA site-selectivity of this agent and also indicated the involvement of the flanking sequences of the hairpin oligonucleotide. In addition, for Co(III)•bleomycin-B_2 the established *cleavage* site-selectivity for 5´-GT and 5´-GC sites was correlated to drug-DNA association in this *binding-only* assay. Our results also suggest a preferred, and to date unreported, *tetranucleotide* site-selectivity for metallobleomycin involving two drug equivalents and cross-strand, “back-to-back” 5´-GT and 5´-GC sites such as 5´-ACGT and 5´-ACGC.

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Cul-4A targets p27 for degradation and regulates proliferation, cell cycle exit, and differentiation during erythropoiesis.

Binghui Li, Nan Jia, Yahaira Naaldjik, Reuben Kapur, and Kristin T. Chun
Departments of Pediatrics Biochemistry & Molecular Biology, Herman B Wells Center for Pediatric Research, Indiana University School of Medicine, Indianapolis, IN

As erythroid progenitors differentiate into precursors and finally mature red blood cells, they induce the expression of lineage-specific genes, and their proliferation declines until cell cycle exit. *CUL-4A* encodes a core subunit of a ubiquitin ligase that targets proteins for ubiquitin-mediated degradation, and *CUL-4A* haploinsufficient mice display hematopoietic dysregulation with fewer multipotential and erythroid committed progenitors (3-fold and 5-fold, respectively). In this study, stress induced by 5-fluorouracil or phenylhydrazine reveals a delay in the ability of *CUL-4A* +/- mice to recover erythroid progenitors and precursors and to reestablish normal hematocrits. Conversely, over-expression of *CUL-4A* in a growth factor-dependent, proerythroblast cell line increases proliferation 34% and the proportion of cells in S-phase 8%. When proerythroblasts are induced to terminally differentiate, endogenous *CUL-4A* protein expression declines 3.6-fold. Its enforced expression interferes with erythrocyte maturation and cell cycle exit, and instead promotes proliferation. Furthermore, p27 accumulates during erythroid terminal differentiation, but *CUL-4A* enforced expression increases p27 protein turnover nearly 7-fold and attenuates its accumulation. *CUL-4A* and p27 proteins co-immunoprecipitate, indicating that a Cul-4A ubiquitin ligase targets p27 for degradation. These findings suggest that a Cul-4A ubiquitin ligase positively regulates proliferation by targeting p27 for degradation and that *CUL-4A* down-regulation during erythroid terminal differentiation allows p27 to accumulate and signal cell cycle exit.

Sphingosylphosphorylchoiline (SPC) inhibits B16 tumor growth *in vivo*

Hui Li [1,2], Yijin Xiao [2,3] ,Ying Jiang, [2] Juan Ren[2] and Yan Xu[1,2]

[1]Division of Gynecologic Oncology, Department of Obstetrics and Gynecology, Indiana University Cancer Center, Indiana University School of Medicine, 975 W. Walnut St. IB355A, Indianapolis, Indiana; [2]Department of Cancer Biology, Lerner Research Institute, The Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, OH 44195; [3]Frontage Laboratories, Inc., 105 Great Valley Parkway, Malvern, PA 19355;

We and others have shown previously that a bioactive lysophospholipid, sphingosylphosphorylcholine (SPC), promotes cell proliferation in non-malignant cells, but inhibits cell proliferation in many different cancer cells, including ovarian, breast, prostate and colon cancer cell lines, suggesting that SPC might be a useful therapeutic agent. To determine whether SPC has a tumor suppression role *in vivo,* we used a mouse model with B16-F10 cells subcutaneously injected. We injected ten million cancer cells subcutaneously in each of the two flanks of 8 immunocompetent mice, and to these 8 mice, PBS, 0.1, 0.5, 0.8, or 1 mg of SPC (dissolved in PBS) was co-injected into one of the two flanks in each mouse. We observed a dramatic effect of SPC-induced tumor killing. Tumors developed in the flanks without SPC treatment in all 8 mice. In contrast, tumor growth was SPC-dose-dependently inhibited in the treated flank, and a completely eradicated tumor growth was achieved between 0.8-1.0 mg of SPC. In the flanks injected with the highest dose of SPC (1.0 mg), the tissues surronding the injection site appeared normal and no toxicity was detected. In the same set of mice, tumors grew quickly in the other untreated flanks, and the highest dose of SPC (1.0 mg) may have some inhibitory effects on the tumor growth in the other flank. Analysis of SPC in mice after SPC administration suggests that SPC degraded rather quickly in mice. We are in the process of studying the molecular mechanisms of SPC's effect *in vitro*.

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*Corresponding author: Division of Gynecologic Oncology, Department of Obstetrics and Gynecology, Indiana University Cancer Center, Indiana University School of Medicine, 975 W. Walnut St. IB355A, Indianapolis, IN 46202. xu2@iupui.edu

The NCX1 Knockout Mouse as a Model for Studying The Role of Circulation in Distributing Mammalian Yolk Sac Definitive Hematopoietic Progenitor Cells to the Embryo Proper

Christopher Lux[1], Kathleen McGrath[2], Simon Conway[1], James Palis[2], Momoko Yoshimoto[1], Mervin C. Yoder[1]. [1]*Dept. of Pediatrics, Wells Center for Pediatric Research, Indiana University School of Medicine, Indianapolis, IN;* [2]*Dept. of Pediatrics, University of Rochester School of Medicine, Rochester, NY.*

The yolk sac is the lone site of primitive hematopoiesis. The role of the yolk sac in generating definitive hematopoietic progenitors, however, has remained controversial. One complicating factor preventing an accurate investigation of this subject has been the onset of early circulation which alters the localization of hematopoietic progenitors. An Ncx1 knockout mouse (which fails to initiate a heartbeat) is used here to investigate the temporal and spatial distribution of definitive hematopoietic progenitor cells (HPCs: adult type BFU-E, CFU-GM, CFU-GEMM) in an environment lacking circulation. Embryos were harvested from timed pregnancies from Ncx1 heterozygote crosses beginning at the onset of circulation 8.5 days post conception (E8.5) and ending 36 hours later (E10). Developmental age was determined by somite pair number. All embryos were carefully separated from their yolk sacs and both samples were digested and plated in methylcellulose using a previously published definitive hematopoietic progenitor colony assay. Colonies were counted at seven days and then collected for genotyping. In embryos of all genotypes at E8.5, definitive HPCs were enriched more than 28 fold in the yolk sac compared to the embryo proper (EP), but following redistribution by a functional circulation in E10 wild type and heterozygous embryos, the ratio drops to a 3 fold enrichment in the yolk sac (similar to the 3-5 fold enrichment previously reported at this age; KM & JP, Blood 2003). Ncx1 null embryos lacking circulation produced few HPCs in the EP and never redistribute the HPCs as late as E10 resulting in a 72 fold enrichment in the yolk sac even at E10. Whole mount ζ-hemoglobin mRNA staining and benzedine staining were carried out to visualize the distribution of blood cells in the yolk sac and embryo proper in the presence and absence of circulation. The staining pattern in null embryos confirms that cells from the yolk sac blood band remain in the yolk sac and are not found in the EP. Our findings support a model in which the primitive and definitive hematopoietic progenitors are generated in the yolk sac and are only redistributed to the EP upon the onset of circulation. Not only is the yolk sac an important source of definitive HPCs, it is the developing embryo's primary source through E10.

Effect of Race on Vincristine-Associated Neurotoxicity in Pediatric ALL Patients

McCammack KC, Renbarger JL

Background There is significant interpatient variability in vincristine pharmacokinetics and related neurotoxicity. Toxicities are often directly related to the degree of drug exposure, so it is speculated that these two observations may be closely related. It has been established that the cytochrome P450(CYP)3A family of enzymes is primarily responsible for vincristine metabolism in hepatic microsomes and we have shown that CYP3A5 is more efficient than CYP3A4. CYP3A5 is polymorphically expressed with approximately 70% of African Americans and 20% of Caucasians expressing functional enzyme. Thus, one may hypothesize that African Americans (commonly CYP3A5 expressors) will metabolize vincristine more efficiently *in vivo* than Caucasians (commonly nonexpressors), resulting in decreased drug exposure and consequently less vincristine-associated neurotoxicity.
Methods A retrospective analysis of vincristine-related side effects in pediatric patients treated for acute lymphoblastic leukemia was performed. Data were collected and compared between Caucasians (n=98) and African Americans (n=24) to examine the association of CYP3A5 genotype between these two subgroups of patients. In addition to race, age, gender, and disease subtype, we captured highest grade of vincristine-associated neurotoxicity (per NIH CTC version 3.0), number of omitted vincristine doses, number of reduced vincristine doses, cumulative vincristine dose, and disease outcome.
Results We found that 33.7% of Caucasians experienced symptoms consistent with vincristine-related neurotoxicity compared to 12.5% of African Americans ($p<0.05$). Furthermore, 1.1% of total doses administered to Caucasian patients were reduced due to vincristine-related neurotoxicity compared to 0.3% given to African Americans ($p<0.001$). An additional 1.1% of all protocol-indicated doses were missed entirely by Caucasians due to severe vincristine-associated toxicity compared to 0.1% of doses given to African Americans ($p<0.025$). Other trends are present but fail to pass statistical standards of chi-squared analysis, including 18.4% of all Caucasian patients undergoing reduced doses due to vincristine-related toxicity compared to 8.3% of African Americans. A further 13.3% of Caucasians missed doses entirely compared to 4.2% of African Americans.
Conclusions These findings offer firm support for the presence of a hereditary component in interindividual variability in vincristine neurotoxic side effects. They suggest a potentially important *in vivo* role for CYP3A5 as a cause of interpatient variability in vincristine-associated neurotoxicity and metabolism.

Selective metabolism of vinblastine by CYP3A5 *in vitro*

McCammack KC, Dennison JB, Jones DR, Hall SD, Renbarger JL.

Background There is large interindividual variation in vinblastine metabolism *in vitro* and *in vivo*. The role of cytochrome P450(CYP)3A enzymes governing vinblastine conversion has been established, allowing for the postulation that genetic polymorphisms that influence CYP3A expression may contribute to this interindividual variability.
Methods We used cDNA-expressed CYPs and a human liver microsome bank to analyze *in vitro* vinblastine metabolism. In addition, we performed inhibition studies to confirm involvement of specific CYP isoforms in vinblastine metabolism.
Results Early analysis demonstrated CYP3A4 and CYP3A5 as the only CYPs to result in significant vinblastine loss producing three main metabolites, metabolite 1 (M1) most abundant among them. Studies with isoform-specific inhibitors confirmed the primary role of the CYP3A subfamily. Recombinant CYP3A5 produced M1 more efficiently than CYP3A4 (approximately 3-fold higher intrinsic clearance for CYP3A5). M1 formation was differentially enhanced by co-expressed cytochrome b5 (approximately 1.5-fold for CYP3A4 and 3.5-fold for CYP3A5) resulting in a 6-fold higher intrinsic clearance for co-expressed CYP3A5+b5. Exogenously added cytochrome b5 had a slight but augmentative effect on both CYP3A4 and CYP3A5 activity. Human liver microsomes with functional 3A5 (*1*1/*1*3 individuals with comparable CYP3A4 activity) were routinely more efficient at producing M1 than were nonexpressing HLMs (*3*3 individuals) with comparable CYP3A4 levels.
Conclusions Our findings reveal that vinblastine is more efficiently metabolized *in vitro* by CYP3A5 than CYP3A4. As such, CYP3A5 genetic polymorphisms may partially account for the interindividual differences observed in the systemic clearance of vinblastine *in vivo*.

Gleevec Resistant Activating Mutation of *c-Kit (D816V)* Demonstrates Ligand Independent Growth and Promiscuous Cooperation with Multiple Cytokine Receptors Via the p85α Subunit of Class IA PI-3Kinase.

Veerendra Munugalavadla, Emily Sims, Jovencio Borneo, Reuben Kapur Pediatrics, Indiana University School of Medicine, Herman B Wells Center for Pediatric Research, Indianapolis, IN, USA

Gain of function mutations in c-Kit are associated with a number of cancers in humans, including gastrointestinal stromal tumors (GIST), mastocytosis and acute myeloid leukemia (AML). Although, gleevec is a potent inhibitor of c-Kit juxtamembrane mutants that are associated with GISTs, little is known about the drugs that inhibit the activity of c-Kit with mutations in the catalytic domain (i.e. substitution of valine for aspartic acid in codon 816: *c-Kit D816V*), which are associated with mastocytosis and/or AML. Although, *in vitro* expression of c-Kit D816V in transformed cell lines results in ligand independent survival due to constitutive phosphorylation, the functional manifestation of this mutation in primary stem/progenitor cells (HSC/Ps) or in mast cells is poorly understood. In addition, little is known about the signaling pathways that contribute to c-Kit D816V induced transformation in HSC/Ps or mast cells. Here, we assessed the role of *c-Kit D814V* in primary bone marrow derived HSC/Ps and mast cells. Consistent with prior reports, expression of *c-Kit D814V* in HSC/Ps resulted in constitutive autophosphorylation of the c-Kit receptor. To determine the affect of this mutation on HSC/Ps, we first assessed alteration in the differentiation of HSC/Ps by assessing the cell surface expression of Gr-1, Mac-1 and F4/80. Compared to HSC/Ps transduced with the empty vector, no significant perturbation in the expression of Gr-1, Mac-1 or F4/80 was observed in *c-Kit D814V* expressing HSC/Ps (n=3, $p<0.5$). Consistent with the constitutively active nature of *c-Kit D814V*, a 20 to 40 fold increase in the growth of c-Kit D814V expressing HSC/Ps was observed in the absence of ligand stimulation as assessed by thymidine incorporation and colony forming ability (n=3, $p<0.005$). The growth of *c-Kit D814V* expressing HSC/Ps was further augmented 3 to 5 fold in the presence of stem cell factor (SCF), the ligand for c-Kit, compared to controls. Interestingly, stimulation of *c-Kit D814V* expressing HSC/Ps with additional cytokines, including G-CSF, M-CSF or IL-3, either alone or in combination with SCF further augmented their growth compared to controls. In line with the promiscuous cytokine induced cooperation via *c-Kit D814V* in HSC/Ps, expression of *c-Kit D814V* in primary mast cells also resulted in a significant increase in their growth via IL-3, IL-4 and IL-10; three cytokines known to play an essential role in mast cell growth and differentiation. We and others have shown that wildtype c-Kit stimulation by SCF results in the activation of the PI-3Kinase pathway via the recruitment of p85α regulatory subunit of class IA PI-3Kinase to the receptor. Since p85α is constitutively bound to *c-Kit D814V*, we hypothesized that p85a may contribute to *c-Kit D814V* induced hyperactivation and cytokine induced promiscuous growth in HSC/Ps and mast cells. To test this, we expressed *c-Kit D814V* in *p85α-/-* HSC/Ps and mast cells. Remarkably, deficiency of p85α in the setting of *c-Kit D814V* rescued ligand independent growth mediated via *c-Kit D814V* to near wildtype levels (n=3). More importantly, the promiscuous and enhanced growth in combination with other cytokines seen in *c-Kit D814V* expressing HSC/Ps and mast cells was corrected to wildtype levels. Taken together, our results identify p85α regulatory subunit of class IA PI-3Kinase as a novel therapeutic target for the treatment of mastocytosis and AML involving the *c-Kit D814V*.

A Novel Histone Deacetylase Inhibitor with Potent Antiproliferative Activity Against Ovarian Cancer

Michael Mand[1], Yating Yang[3], Vasu Tumati[1], Ceazon Edwards[1], Samuel Kulp[3], Ching-Shih Chen[3], Kenneth Nephew[1,2], and Curtis Balch[1,2]

[1]Medical Sciences, Indiana University School of Medicine, Bloomington, IN
[2]Indiana University Cancer Center, Indianapolis, IN
[3]Division of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, The Ohio State University, Columbus, OH

Histone deacetylase (HDAC) inhibitors have now shown antineoplastic activity against a number of hematologic and solid malignancies. However, as most agents now in clinical trials were not initially discovered as HDAC inhibitors (HDACIs), enzyme inhibition is suboptimal, resulting in variable activity and potency. In this study, the widely used HDACI suberoylanilide hydroxamic acid (SAHA) was compared to a novel HDACI, HDAC42, which was rationally designed for optimal insertion into the HDAC active site "pocket." In 3 of 4 ovarian cancer cell lines examined, HDAC42 effective doses (IC50s) were similar to or lower than those of SAHA. In normal surface ovarian epithelial cells, HDAC42 possessed an IC50 of 8.9 μM, less toxic than SAHA (IC50 of 6.3 μM). Biochemically, HDAC42 was more potent than SAHA, at 1 μM for 24 h, of enhancing acetylation of both histone (histone H3) and nonhistone (α-tubulin) proteins in the ovarian cancer cell line CP70. The mechanism of HDAC42 growth inhibition was primarily through apoptotic pathways, as demonstrated by three separate assessments (Annexin V/PI assay, flow cytometry, and PARP cleavage). Additionally, HDAC42 likely exerts its effects through both p53-dependent cascades, as shown by upregulation of *NOXA* (3-fold), and p53-independent cascades, as demonstrated by induction of γ-*globin* (45-fold), in the p53-positive cell line A2780. The p53 target gene *p21*, by contrast, was upregulated in both p53-negative and –positive cells, suggesting that p53 was not fully necessary for induction. In mice, HDAC42 was capable of resensitizing xenografts of the platinum-resistant cell line CP70 to cisplatin, with greater efficacy than SAHA; 50 mg/kg HDAC42 Q2D plus 6 mg/kg cisplatin weekly completely abrogated tumor growth. In summary, this unique HDAC inhibitor is a promising preclinical candidate for the treatment of human ovarian cancer.

The Platelet Derived Growth Factor Receptor is Destabilized by Geldanamycins in Cancer Cells

Daniela E Matei , Minati Satpathy, Yi-Chun Lai, Harikrishna Nakshatri, David B. Donner

The heat shock protein HSP90 serves as a chaperone for receptor protein kinases, steroid receptors and other intracellular signaling molecules. Targeting HSP90 with ansamycin antibiotics disrupts normal processing of clients of the HSP90 complex. The PDGFRα is a tyrosine kinase receptor upregulated and activated in several malignancies. Here we show that the PDGFRα forms a complex with HSP90 and the co-chaperone cdc37 in ovarian, glioblastoma and lung cancer cells. Treatment of cancer cell lines expressing the PDGFR with the HSP90 inhibitor 17-allylamino-17-demethoxygeldanamycin (17-AAG) promotes degradation of the receptor within 3 hours. Likewise, phospho-Akt is degraded after treatment with 17-AAG. In contrast, PDGFRα expression is not affected by 17-AAG in normal human smooth muscle cells (HSMC) or in murine fibroblasts (NIH 3T3). PDGFRα degradation by 17-AAG is inhibited by the proteasome inhibitor MG132. High molecular weight, ubiquitinated forms of the receptor are detected in cells treated with 17-AAG and MG132. Degradation of the receptor is also inhibited by a specific neutralizing antibody to the PDGFRα, but not by a neutralizing antibody to PDGF or by Gleevec. Ultimately, PDGFRα mediated cell proliferation is inhibited by 17-AAG. These results show that 17-AAG promotes PDGFR degradation and inhibits PDGF-induced cancer cell proliferation, suggesting a potential therapeutic role for HSP90 inhibitors in malignancies dependent on the PDGFR. This study shows that not only mutated tyrosine kinases, but also overexpressed receptors can be targeted by 17-AAG.

Alterations in DNA Methylation Patterns Associated with Onset of Chemotherapy Resistance in Ovarian Cancer

John S. Montgomery, Curtis Balch, Mike Mand, Yoo-Sung Kim, Henry H. Paik, Sun Kim, Tim H-M. Huang, and Kenneth P. Nephew. *Indiana University, School of Medicine, Bloomington, IN; The Ohio State University, Columbus, OH, Inha University, Incheon, Korea.*

Aberrant methylation of promoter CpG islands is a well-documented tumor suppressor silencing mechanism that occurs in malignancy progression. Additionally, the onset of resistance to specific chemotherapeutics, such as the DNA-crosslinking agent cisplatin, has been linked with increased DNA methylation of specific genes, including *hMLH1*, which encodes a mismatch repair enzyme associated with apoptosis. To assess alterations in DNA methylation correlated with cisplatin resistance, including possible mechanisms, we utilized a commercially available methylation microarray of 82 gene promoters (Panomics, Redwood City, CA) to determine "methylation signatures" for cisplatin-sensitive A2780, cisplatin-resistant CP70, and normal ovarian surface epithelial (NOSE) cells. The methylation signatures of NOSE and A2780 cells were highly similar, with 13 methylated and 68 unmethylated genes in common. In contrast, CP70 cells possessed 24 genes exclusively methylated, including *hMLH1,* suggesting increased methylation associated with the chemoresistant phenotype. To determine possible mechanisms of cisplatin-associated DNA methylation, we developed a cell culture model system. Single cells of cisplatin-sensitive A2780 were isolated and expanded. Chemosensitive clones were treated with variable doses of cisplatin, followed by assessment of cell number (by MTT assay) and determination of the 50% growth inhibitory dose (IC50). Each subsequent cycle entailed selection of resistant cells using the 70% growth inhibitory dose (IC70) of cisplatin, followed by redetermination of the IC50 values of the surviving cells. Following six cisplatin treatment cycles, IC50 doses increased from an average of 5 μM to 30 μM. To examine changes in gene promoter methylation status, genomic DNA was isolated following each selection cycle and examined using Differential Methylation Hybridization (DMH), a comprehensive methylation microarray approach. Genes exhibiting a 2-fold change in methylation were used as input for a pattern discovery algorithm (MEME).. The output of MEME is a set of consensus sequences that are currently being validated for their ability to distinguish methylation-prone and methylation-resistant genes associated with cisplatin resistance. In summary, we demonstrate that DNA methylation plays a significant role in the development of platinum resistance in ovarian cancer cells. Further studies are now underway to determine possible mechanisms and strategies for chemosensitization.

[^{18}F]Fluoromethylallylcholine (FMAC): Synthesis and Evaluation of a New PET Tracer for Cancer Imaging.

Rachid NAZIH, Aditya BANSAL, Shuyan Wang, Timothy DEGRADO
Department of Radiology, Indiana University School of Medicine, 1345 W. 16th Street L-3 R# 202, Indianapolis, IN 46202, U.S.A

Keywords: Fluoromethylallylcholine, PET, Cancer Imaging.

Objectives: ^{18}F-labeled choline analogs are currently under development as oncologic probes for PET. In this study, [^{18}F]fluoromethylallylcholine (FMAC) was synthesized and preliminarily evaluated as a new PET imaging tracer for cancer imaging. Methods: FMAC was synthesized using an automated system (Tracera LLC, Zionsville, IN) via the alkylation of *N*-allyl-*N*-methyl-*N*-hydroxyethylamine (10 mg) with [^{18}F]fluoromethyl triflate in acetone (0.4 mL). The product was held up on a cation-exchange cartridge, isolated from the precursor using rinses with ethanol and water, and eluted form the cartridge with sterile isotonic saline. Radiochemical purity was determined by cation-exchange HPLC. Biodistribution and metabolism studies were performed in Fisher rats bearing subcutaneous 9L-glioma tumors in comparison with [^{14}C]choline and [^{18}F]fluorocholine (FCH). Results: Radiochemical yields of FMAC were found to depend on temperature, reaction time and concentration of amine precursor. Optimal [^{18}F]fluoromethylation yields of up to 90% were obtained at reaction temperatures of 30-40 °C. At higher temperatures decomposition of the product was observed. High radiochemical purity (99%) of FMAC was achieved. In 9L-glioma-bearing rats, highest uptake was seen in kidneys and liver. In comparison with FCH and [^{14}C]choline, FMAC showed slower oxidation to the corresponding betaine analog in lung, liver and kidney which lead to a prolonged plasma clearance of nonmetabolized FMAC. Tumor:blood and tumor:muscle ratios for FMAC were 10 and 2 fold higher, respectively, than seen with FCH. Uptake of FMAC in kidney was lower than for [^{14}C]choline and FCH, while urinary clearance was increased. Conclusions: FMAC is an ^{18}F-labeled choline analog with slower oxidation, more prolonged plasma clearance, and higher tumor:background ratios than FCH. These results encourage further evaluation of FMAC as a cancer imaging probe for PET.

Combining NMR and MS-based Metabolomics for Cancer Detection in Mice

Zhengzheng Pan, Nari Talaty, Graham Cooks and Daniel Raftery

Department of Chemistry, Purdue University, West Lafayette, IN, 47907

Metabolomics is of increasing interest in the life sciences because it offers an approach that gives information on a whole organism's functional integrity over time, including changes following exposure to drugs or toxic, environmental stimulants. This new approach to cancer diagnosis utilizes NMR- and MS- based metabolomics, which measures the metabolic levels in biofluid samples. With multivariate statistical analysis, such as principal component analysis (PCA), differences between samples are thus emphasized and the identification of potential biomarkers is achieved.

NMR- and MS- based metabolomics were applied to the detection of lung cancer in mice by measuring urine samples without preparation or separation. High-resolution NMR spectroscopy measures the complex biofluid samples quantitatively and reproducibly. NMR provides high-reproducibility and thus a reliable evaluation of the sample. High sensitivity and specificity of desorption electrospray ionization (DESI) mass spectrometry requires no sample preparation. Multivariate statistical analysis, especially principal component analysis is used to differentiate sick and healthy subjects. It is proven that both NMR and DESI-MS give a similar pattern of classification between different samples. The combination of NMR and DESI-MS provides a promising new approach to the detection of cancer. In addition, by using loading plots from PCA results, potential biomarkers are collected and identified.

A group of common compounds from the loading plots of NMR and DESI-MS results are used to perform selective monitoring of cancer. Reduced PCA analysis based on these common compounds gives similar separation among samples. It is proven that detection of cancer can be reduced to a group of important compounds (biomarkers) which may make the diagnostic process easier and less time-consuming. Further validation of these compounds is currently underway.

Influence of DNA sequence bias/strand orientation and redox effect on proteins involved in DNA double-strand break repair

K.S. Pawelczak, B.J. Andrews, J.A. Lehman and J.J. Turchi

Department of Medicine, Division of Hematology/Oncology, Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, IN 46202

Non-homologous end joining (NHEJ) is the major pathway involved in the repair of ionizing radiation (IR) induced DNA double strand breaks. The specifics of the NHEJ pathway are not fully characterized and understanding more clearly the mechanisms of NHEJ could potentially illuminate a favorable target for the treatment of a variety of cancers. The DNA-dependent protein kinase catalytic subunit (DNA-PKcs) and Ku are essential components of the complex that catalyzes NHEJ. We have investigated the effect of DNA strand orientation and sequence bias on the activation of DNA-PK. DNA-PK kinase activity was measured and the results revealed that DNA strand orientation and sequence bias select for 3´ pyrimidine-rich strands which dramatically stimulate kinase activation. In addition, the anticancer agent cisplatin, which forms covalent adducts on DNA, resulted in differing degrees of inhibition depending on position and distance from the terminus, as well as strand orientation. Independent of the sequence specificity/strand bias findings, we determined that redox status has a significant effect on the intrinsic nature of Ku binding to DNA. Pre-steady state kinetic analysis revealed that Ku binding to DNA under limited reduction followed a two-step binding process, fully reduced conditions followed one-step binding kinetics and completely oxidized Ku was undetectable with this method. In addition, the differences observed under the varying redox conditions could not be attributed to multiple Ku's binding or the inward sliding of Ku on the DNA. Studies done to determine Ku dissociation rates indicated a dramatic difference as a function of redox. This effect was induced by diamide treatment and reversed by DTT treatment, demonstrating a reversible redox effect on the stability of the Ku-DNA complex. To test the hypothesis that Ku could undergo a redox-dependent conformational change, limited proteolysis combined with MALDI-TOF and LC-Q tandem mass spectrometry was employed and revealed a redox-sensitive area in the ring structure where DNA-sliding takes place. To further characterize the redox-sensitive areas of Ku, a chemical modification procedure using NHS-biotin to target lysines was used to measure solvent-accessibility changes in the conformation. Differential labeling of lysines for oxidized and reduced Ku has again signified a conformational change in this protein based on redox. In light of incorrectly or unrepaired DNA double-strand breaks likely leading to genomic instability therefore progessing to cancer, our biochemical findings on proteins that repair this pathway are of great significance.

2-Methoxyestradiol-resistant cancer cells overexpress functional laminin α4.

Yesim Gökmen-Polar, Kerry L. Sanders, Jonathan C.R. Jones, Harikrishna Nakshatri and George W. Sledge, Jr. *Department of Medicine, Indiana University School of Medicine, Indianapolis, IN, Department of Cell and Molecular Biology, The Feinberg School of Medicine at Northwestern University, Chicago, IL.*

2-methoxyestradiol (2ME2), an estrogen metabolite with antiproliferative and antiangiogenic activity, binds tubulin at the colchicine site and depolymerizes microtubules. We have previously generated a stable 2ME2-resistant (2ME2R) MDA-MB-435 human cancer cell line and shown that beta-tubulin mutations are associated with resistance to 2ME2 (Cancer Res 65:9406-14, 2005). In the present study, to further identify molecular mechanisms involved in 2ME2 resistance, we performed Atlas cDNA Cancer microarrays between 2ME2R and the more sensitive parental cell line (P435). Within the set of differentially expressed genes, we have specifically focused on the laminin α4 subunit, as laminins can enhance cell adhesion, tumorigenicity and contribute to drug resistance. We have confirmed the overexpression of laminin α4 subunit in 2ME2R cells by Northern analysis. We also have demonstrated that 2ME2R cells secrete significant amount of laminin α4 subunit. To understand the adhesive nature of 2ME2R cells, we have first determined the adhesion ability of 2ME2R cells to laminin 1 and fibronectin representing the integrin-mediated cell adhesion or poly-L-lysine, which does not require integrins. 2ME2R cells exhibit dose-dependent, enhanced adhesion to laminin 1 compared to parental cells, while they have lower adhesion to fibronectin than parental cells. In addition, 2ME2R cells also adhere better to poly-L-lysine suggesting the additional involvement of integrin-independent signaling. There is evidence that specific laminin isoforms can assembly to each other. If a mixture of one or more polymerizing laminin isoforms exists in a basement membrane, laminins can form a cooperative network. To determine whether the presence of laminin α4 subunit in 2ME2R cells can enhance laminin1 adhesion in resistant cells, we treated the cells with an antibody (2A3) against the G domain of the laminin α4 subunit. We demonstrate that functional blocking of laminin α4 reduces adhesion of 2ME2R cells to laminin at a significant level. This suggests that laminin α4 is necessary for the adhesion of 2ME2R cells to laminin-1. Further studies will address the nature of laminin α4-laminin 1 adhesion-mediated signaling in 2ME2R cells and the contribution of laminin α4 subunit to 2ME2 resistance.

Acrylonitrile induces Oxidative DNA damage in rat glial cells

Xinzhu Pu, Lisa M. Kamendulis, and James E. Klaunig
Indiana University School of Medicine, Division of Toxicology, Indianapolis, IN

Acrylonitrile (ACN), used in the manufacture of plastics, acrylic fibers, and synthetic rubber, resulted in a dose-related increase in gliomas in rat following chronic exposure. Studies support that the mechanism of ACN carcinogenicity involves induction of oxidative stress. Metabolism of ACN occurs through glutathione (GSH) conjugation and oxidation by cytochrome P450, with the latter pathway producing a reactive epoxide, cyanoethylene oxide (CEO) that can further undergo metabolism to cyanide. Previous studies have reported increased oxidative DNA damage following ACN exposure, however, the source of the oxidative damage has not been demonstrated. Further, the potential of ACN or metabolite (e.g. CEO) to induce direct DNA damage as a mechanism for ACN carcinogenicity has been questioned. The present study examined the ability of ACN to induce DNA damage in the DITNC1 rat glial cell line using the alkaline Comet assay. Oxidized DNA damage was also investigated using the formamidopyrimidine DNA glycosylase modified Comet assay. No increase in direct DNA damage was seen in glial cells exposed to sublethal concentrations of ACN (0-1.0 mM) for 24 hrs. However, ACN resulted in a concentration-related increase in oxidative DNA damage after 24 hrs. Antioxidant supplementation (α-tocopherol, (-)-epigallocathechin-3 gallate, or Trolox) reduced ACN-induced oxidative DNA damage. Additional studies were conducted to identify which pathway of ACN metabolism contributed to increased oxidative DNA damage. Depletion of GSH with 0.1 mM buthionine-sulfoximine increased ACN-induced oxidative DNA damage (22-46%), while co-treatment with a GSH precursor, L-2-oxothiazolidine-4-carboxylic acid (2.5 mM), reduced ACN-induced oxidative DNA damage (7-47%). Co-treatment with 0.5 mM 1-aminobenzotriazole, a cytochrome P450 inhibitor, prevented the oxidative DNA damage by ACN. Cyanide (0.1-0.5 mM) increased oxidative DNA damage (44-160%) in glial cells. Taken together, these results suggest that ACN-induced oxidative DNA damage arises mainly through the P450 metabolic pathway, and GSH depletion may be a contributory factor.

Expression and function of Toll-like receptors on $CD_4^+CD_{25}^+$ $FoxP3^+$ Regulatory T cells

Peng Qu, Abigail Weliver, Pratibha Singh, Wei Li, Cheong-Hee Chang and Soon-Cheol Hong

Department of Microbiology and Immunology, and the Walther Oncology Center,
Indiana University School of Medicine, Indianapolis, Indiana 46202.
Walther Cancer Institute, Indianapolis, Indiana 46202.

Tel: 317-274-7560, Fax: 317-274-7592, E-mail: sohong@iupui.edu

Regulatory T cells (Tregs) play a major role in immune regulation of autoimmunity and graft rejection. We discovered the expression of Toll-like receptors (TLRs) on a subpopulation of natural Tregs. Approximately 10% of Tregs from spleen and more than 40% of Tregs in bone marrow express TLRs. TLR^{hi} Tregs differ from $TLR^{lo/-}$ Tregs in their expression of several Treg markers. TLR^{hi} Tregs express higher levels of CD_{45RB} and CD_{38}, while $TLR^{lo/-}$ Tregs express low levels of CD_{45RB} and CD_{38}. Other markers, CD_{152}, GITR and CD_{62L}, are equally expressed on all Tregs. While both TLR^{hi} and $TLR^{lo/-}$ freshly isolated Tregs suppress proliferation of responder T cells, their response to TLR ligand separates these two Tregs. In response to LPS or TCR stimulation, TLR^{hi} Tregs, but not $TLR^{lo/-}$ Tregs, proliferate and lose their expression of FoxP3. These data indicate that there exist two different Tregs that respond differentially to various stimulations.

Vα14^{+} NKT cell-dependent antitumor activity in a murine B cell lymphoma model

Gourapura J. Renukaradhya, Masood Khan, Marcus Vieira, and Randy R. Brutkiewicz

Department of Microbiology and Immunology,
Indiana University School of Medicine and the Walther Oncology Center,
Indianapolis, IN 46202 and the Walther Cancer Institute, Indianapolis, IN 46208.

CD1d is an MHC class I-like molecule that presents lipids to a unique subpopulation of T cells called natural killer T (NKT) cells. NKT cells are an immunoregulatory T cell subpopulation known to secrete both Th1 and Th2 cytokines upon activation. Vα14^{+} and Vα14^{-} NKT cell subpopulations can mediate distinct antitumor immune responses in some tumor models. The role of NKT cells and CD1d molecules in the immune response against hematopoietic tumors is still largely unexplored. We used the murine B-cell lymphoma cell line, NS0, which lacks detectable CD1d expression, in the study described here. Mouse CD1d1 cDNA or empty vector was transfected into NS0 cells to generate CD1d^{+} (NS0-CD1) and control (NS0-V) cell lines, respectively. These were inoculated i.p. into syngenic wildtype, CD1-deficient (lack both Vα14^{+} and Vα14^{-} NKT cells; CD1KO), and J 18KO (lack only Vα14^{+} NKT cells) BALB/c mice. It was found that less than 30% of the wildtype or CD1KO mice developed ascites tumors with either cell line. Interestingly, over 60% of Jα18KO mice bearing NS0-V cells ($P<0.05$), and 100% of the Jα18KO mice inoculated with NS0-CD1 cells succumbed to the tumor ($P<0.0001$), confirming a role for NKT cell-dependent antitumor activity. Adoptive transfer of Vα14^{+} NKT cells to NS0-CD1-bearing Jα18KO mice significantly reduced tumor development. Overall, therefore, these data suggest that Vα14^{+} NKT cells mediate a protective antitumor effect in this B-cell lymphoma model, and point to a potential novel target to be exploited in the treatment of such tumors.

A Role for Duplin in SRF-mediated Signaling

Jennifer M. Rodenberg and B. Paul Herring, PhD
Indiana University School of Medicine, Department of Cellular and Integrative Physiology

Differentiated smooth muscle cells have the remarkable ability to switch between a quiescent contractile phenotype and a proliferative, synthetic phenotype under many pathological conditions. Serum response factor (SRF) is a transcription factor that is crucial for regulating genes characteristic of contractile smooth muscle cells as well as activating genes required for cell proliferation. The ability of SRF to regulate these two disparate phenotypes is dependent on its ability to interact with proteins that alter its activity towards differentiation genes as opposed to proliferation genes. Identifying and unraveling the function of these SRF-associated proteins is thus crucial for understanding the mechanisms regulating the phenotype of smooth muscle cells. A yeast two-hybrid screen using SRF as bait identified the protein Duplin. Duplin is a nuclear protein that was previously shown to bind to -catenin and inhibit its signaling. Interestingly, knocking out Duplin is embryonic lethal, due to defects in gastrulation, although expression of -catenin target genes was unaffected (Nishiyama, et al. 2004). SRF-GST fusion proteins demonstrate a direct interaction of SRF with the amino-terminal portion of Duplin. Preliminary results from mammalian two-hybrid experiments in cos cells also indicate a direct interaction between SRF and Duplin. The phenotype of SRF knockout mice is similar to the Duplin knockout mice with a failure of mesoderm formation during gastrulation. Together these data suggest that Duplin may play an important role in modulating SRF-dependent gene expression. shRNA is currently being used to knock-down Duplin expression in smooth muscle and nonmuscle cells to determine the effects on SRF-dependent genes involved in differentiation and growth. Although much of the physiological significance of Duplin remains unknown, from our preliminary data we can conclude that Duplin binds SRF independently of possible interactions with -catenin.
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Transglutaminase 2 is overexpressed in ovarian cancer and facilitates the adhesion of cancer cells to fibronectin

Minati Satpathy, Daniela Matei, MD
Indiana University School of Medicine Division of Hematology Oncology

Background: Tissue transglutaminase (TG2) is an enzyme involved in Ca^{++}-dependent aggregation and polymerization of proteins through cross-linking of glutamine residues. Recent reports have noted increased TG2 expression in epithelial malignancies. Using microarray hybridization, we found that TG2 is over-expressed in ovarian cancer cells compared to normal surface ovarian cells (11-fold difference, *p*-value=0.0000). The role of TG2 in epithelial ovarian cancer is not known.

Materials and Methods: Western Blotting and immunohistochemistry were used to determine TG2 expression in ovarian tumors and in ascites fluid from patients with ovarian cancer. Adhesion to fibronectin (FN) was measured using a solid-phase adhesion assay in ovarian cancer cells.

Results: TG2 was detected in 8 of 10 ovarian tumors at protein level, but not in 3 samples of normal ovarian tissue. TG2 expression was detected by immunohistochemistry in 65% of 26 ovarian tumors. In contrast, none of the 6 normal ovarian samples stained for TG2. TG2 is a secreted protein and was detected in 25 of 30 specimens of peritoneal fluid collected from patients with ovarian cancer. In contrast small amounts or no TG2 was found in ascites fluid from patients with inflammatory conditions.

To determine the function of TG2 in human ovarian epithelial cells, the protein was knocked down by stable transfection with an anti-sense TG2 construct (SkOv3 cells) and was stably overexpressed using WT TG2 cloned in pCDNA3.1 (OV90 cells). Adhesion to fibronectin was decreased by 50% in SKOV3 cells lacking TG2 compared to cells transfected with pCDNA3.1. Cells transfected with anti-TG failed to spread on FN, as assessed by immunoflourescent staining for phalloidin and microscopic examination. Similarly, pre-incubation with a neutralizing antibody against the integrin binding domain of TG2 significantly reduced the adhesion of cells to fibronectin (~ 30%). In contrast, stable TG2 overexpression lead to increased adhesion to FN in OV90 cells.

Phosphorylation of the focal adhesion kinase (FAK), engaged upon cell adhesion, was inhibited in cells where TG2 was knocked down. Transient over-expression of TG2 led to increased adhesion-induced FAK phosphorylation. Specific association of TG2 with β1 integrin was demonstrated by co-immunoprecipitation. Knock-down of TG2 lead to decreased levels of β1 integrin, which was reversed in the presence of cyclohexamide and MG132, suggesting that TG2 is necessary for stabilizing β1 integrin.

Conclusion: These data suggest that transglutaminase 2 is expressed in epithelial ovarian tumors, in a cancer-restricted manner. TG2 is necessary for ovarian cancer cell adhesion to the extracellular matrix by binding to and stabilizing the β1 integrin complex.

Loss of Mist1 Sensitizes Cells to the Initiation Events of *Kras*^{G12D} Expression During Pancreatic Cancer Development.

G. Shi, L. Zhu, Y. Sun, D.A. Tuveson[1], C.M. Schmidt[2], R. Hruban[3], and S.F. Konieczny, Department of Biological Sciences and the Purdue Cancer Center, Purdue University, West Lafayette, IN; [1]Departments of Medicine and Cancer Biology, University of Pennsylvania, Philadelphia, PA; [2]Departments of Biochemistry and Molecular Biology, Indiana University Medical School, Indianapolis, IN; [3]Departments of Pathology and Oncology, Johns Hopkins University School of Medicine, Baltimore, MD.

Pancreatic cancer is the fourth leading cause of cancer deaths in the United States with pancreatic ductal adenocarcinoma (PDA) accounting for the majority of pancreatic malignancies. Despite a number of advances in pancreas biology, our understanding of PDA pathogenesis remains incomplete. What is known is that PDA initiates through activating mutations in the *KRAS* protooncogene (usually Kras^{G12D}) that contribute to the formation of precursor lesions known as pancreatic intraepithelial neoplasia (PanIN). Unknown are the identities of the pathways responsible for the unique pathologies of the PanIN and PDA spectrums and the individual cell types that contribute to this disease. In an effort to address these key questions, we have examined the importance of Mist1 - an acinar cell restricted basic helix-loop-helix transcription factor - to pancreatic cancer. Using the *Mist1* locus, the *Kras*^{G12D} oncogene, and homologous recombination, we have generated *Mist1*^{KrasG12D} mice. *Mist1*^{KrasG12D} mice develop a distinct spectrum of pancreatic tumors that exhibit acinar and ductal properties and that lead to premature death by 12 months. Interestingly, the Mist1 protein is an absolute requirement for animal survival since *Mist1*^{KrasG12D} mice lacking *Mist1* succumb to severe pancreatic disruptions at birth. Similarly, replacement of a WT *Mist1* allele with a dominant-negative mutant (Mist1^{MB}) also leads to aggressive pancreas disruptions. To further investigate the role of Mist1 in pancreatic cancer progression, we next introduced the *Mist1* null (*Mist1*^{KO}) genetic background into a less severe pancreatic cancer mouse model in which the *Kras*^{G12D} oncogene is expressed from the endogenous *Kras* promoter in a pancreas-specific manner (*LSL-KR/Cre* mice). Previous studies on *LSL-KR/Cre* animals have shown that these mice develop PanINs that are identical to human PanINs. Interestingly, analysis of *Mist1*^{KO}*/LSL-KR/Cre* mice revealed that their pancreata are greatly enlarged when compared to the pancreata of *Mist1*^{WT}*/LSL-KR/Cre* mice. Histological analyses showed that pancreatic transformation in *Mist1*^{KO}*/LSL-KR/Cre* mice is significantly accelerated with an earlier onset and higher occurrence of PanINs, as well as massive exocrine metaplasia, when compared to *Mist1*^{WT}*/LSL-KR/Cre* mice. Similarly, *Mist1*^{KO}*/LSL-KR/Cre* mice exhibit accelerated tumor-associated characteristics that resemble clinical cases of human acinar cystadenoma. Our working hypothesis is that Mist1 is an essential player in pancreatic acinar cell proliferation and transformation events and that loss of Mist1 sensitizes cells to the initiation events of *Kras*^{G12D} expression. We are currently testing this hypothesis by examining human pancreas tumor samples for alterations in the human *Mist1* locus.

Databases of protein disorder and cancer-associated proteins: DisProt & CancerDisProt

Megan Sickmeier[1], Justin Hamilton[1], Tanguy LeGall[1,2], Marc Cortese[1], Vladimir Uversky[1,2], A. Keith Dunker[1,2], and Ya-Yue Van[2]
[1]Center for Computational Biology and Bioinformatics, Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, IN 46202
[2]Molecular Kinetics, Inc., 6201 La Pas Trail, Suite 160, Indianapolis, IN 46268

Intrinsic disorder has been shown to be significantly more prevalent in cancer-associated proteins than in signaling proteins, eukaryotic proteins from SWISS-PROT and those with solved structures. Disordered regions in proteins confer advantages for molecular recognition, thus providing functional benefits for signaling and regulation. For example, disordered regions can bind their targets with high specificity and low affinity, as well as enabling binding diversity so that a single protein region can interact with numerous partners.

The DisProt database of protein disorder (www.disprot.org) is a database that provides experimentally determined structural and functional information for intrinsically disordered proteins. The current version of DisProt contains 438 disordered proteins, 833 disordered regions and is utilized by over 30 countries across the globe. Of the 438 proteins included in DisProt, 115 are associated with cancer as determined by keyword searches of their descriptions in SwissProt.

CancerDisProt is a refined semi-automatically annotated database of 1787 disordered cancer-related proteins that includes a visual representation of protein-protein interaction regions, both experimental and predicted. The aim of CancerDisProt is to provide target selection data for drug discovery. CancerDisProt is a proprietary database that is accessible by contract with Molecular Kinetics.

Correlating Drug Targets to Protein Network Topology with a Gene Dispensability Parameter.

Submitted By
Arti Singh
MS Bioinformatics
School of Informatics
Indiana University

The enormous amount of protein interaction data has opened doors to new ways of looking at it. In this study human protein interactome data is combined with small molecular drug information to examine new insights of drug development using systems biology. This data is further combined with gene essentiality and lethality data. The interaction networks so generated are compared using different network parameters that let better understanding of the roles of participants of the network. A conglomerate of diseases, **C*ancer*** has been chosen to be examined in this study.

This study was started with 67 genes identified as most influential genes in different types of cancers based on a previous study. We superimposed three different logical datasets (human interactome, drug and drug targets, and cancer genes with a dispensability parameter) from different datasources. We are pursuing study on network parameters with the goal to identify measures for targets from the expanded set of 2481 proteins. The poster also aims at presenting the finding if a correlation exists between essentiality/lethality of a gene, network topology features of its corresponding protein and it's being used as a drug target.

With further study of these networks, correlation between drug targets and network properties is being aimed. The study is aspiring at new ways of discovering drug targets. We expect network analysis of proteins implicated in cancers will bring a new perspective to finding target proteins for new disease treatment and drug discovery. On further expansion, this study has the hope of uncovering mechanisms why certain drugs are more potent or toxic than others in the context of molecular interaction networks.

CD154 expression is limited to the TLR^{hi} Tregs

Pratibha Singh, Abigail Weliver, Peng Qu, and Soon-Cheol Hong
Department of Microbiology and Immunology, and the Walther Oncology Center, Indiana University School of Medicine, Indianapolis, Indiana 46202.
Walther Cancer Institute, Indianapolis, Indiana 46202.

CD154 (CD40L) is a critical for initiating immune responses. CD154 is initially discovered by the presence of mutation in CD154 gene in a hyper IgM syndrome patient due to their deficiency in isotype-switching of immunoglobulin. CD154 is not only essential for activation of all antigen-presenting cells (APCs) but also critical for priming of T cell. The activation of APC is mediated by the CD154-CD40 interaction. CD40 is constitutively expressed in all APCs and is further upregulated after infection through Toll-like receptor (TLR) mediated signal via TRIF dependent manner. CD154, on the other hand, considered an inducible molecule expressed only on activated T cells. Contrary to this current knowledge, we discovered that CD154 expression is limited to the $CD4^{+}CD25^{+}FoxP3^{+}$ regulatory T cells (Treg), in particular on $CD103^{+}$ cells, but not on $CD4^{+}CD25^{-}$ T cells even after activation. The CD154 expression is further upregulated after TLR stimulation, but not after TCR stimulation. Those expanded CD154 T cells lose their suppressive activity while losing their FoxP3 expression. These data provide new insight on how CD154 is involved in immune responses and explains the significance of CD154 expressing $FoxP3^{+}$ regulatory T cells in triggering a variety of immune responses.

Ovarian cancer G-protein-coupled receptor 1 (OGR1) suppresses prostate cancer tumor metastasis.

LIsam Shanjukumar Singh[1,2], Haiyan Tan[2], Michael Berk[2] and Yan Xu[1,2]

[1]Division of Gynecologic Oncology, Department of Obstetrics and Gynecology, Indiana University Cancer Center, Indiana University School of Medicine, 975 W. Walnut St. IB355A, Indianapolis, Indiana; [2]Department of Cancer Biology, Lerner Research Institute, The Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, OH 44195.

Prostate cancer (PC) is the second leading cause of cancer-related deaths in men in the United States. Most of the PC patients die from metastases. Ovarian cancer G-protein-couple-receptor 1 (OGR1) has been identified a receptor for sphingosylphosphorylcholine (SPC), as well as a proton sensing receptor. This receptor also possesses constitutive activity. Expression of OGR1 in prostate cancer metastases is 5-fold less when compared to the non-metastasis tumor tissues. We have tested the effect of OGR1 in an orthotopic prostate cancer model in nude mice. We have established vector- and OGR1-transfected PC-3 cells. OGR1 expression in PC-3 cells suppressed prostate cancer metastasis to many organs, including the liver, the lung, and the spleen. However, OGR1 did not significantly affect primary tumor growth and PC3 cell growth as assessed by *in vivo* BrdU incorporation assays and cell doubling-time assays *in vitro,* respectively. Immunohistochemical staining of primary tumors derived from OGR1 and vector PC3 cells with macrophage specific anti-F4/80 antibody, showed that OGR1 significantly increased macrophage infiltration in the tumor. This result suggests that infiltrated macrophages may be involved in OGR1's role in tumor metastasis suppression. In *in vitro* migration assays using Boyden chambers coated with vitronectin, OGR1 expression significantly inhibited PC3 cells migration. This inhibitory effect was reversed by a dominant negative form of G_i or pertussis toxin (PTX), a specific inhibitor for protein G_i, suggesting that OGR1 may constitutively active G_i. To test further whether OGR1 expression resulted in the production or secretion of a soluble factor(s) that contributed to OGR1's anti-migration property, conditioned media (CM) were collected from both vector- and OGR1-PC-3 cells. We found that CM of OGR1-PC3 cells, but not those from vector-transfected cells, inhibited parental PC3 cells migration, suggesting that a secreted factor is responsible for the inhibitory effect of OGR1. Our initial efforts to identify this factor showed that it was heat-insensitive and future investigation on its nature is undertaken currently. In summary, we provide data to strongly suggest that OGR1 is a novel metastasis suppressor gene (MSG) for prostate cancer. The mechanisms by which OGR1 suppresses metastasis may be related to its constitutive activity to induce macrophage infiltration *in vivo*, its ability to activate G_i, as well as its ability to secrete a soluble autocrine/paracrine factor to inhibit cell migration.

Structure and Biochemical Analysis of PRL-1, a Phosphatase Implicated in Cell Growth, Differentiation, and Invation

Jinpeng Sun

The PRL (*P*hosphatase of *R*egenerating *L*iver) phosphatases constitute a novel class of small, prenylated phosphatases that are implicated in promoting cell growth, differentiation and invation, and represent attractive therapeutic targets for cancer treatment.

Here we describe the crystal structures of PRL-1 as well as the catalytic Cys104 to Ser substituted mutant (PRL-1/C104S) in complex with sulfate. Structural and kinetic analyses provide a molecular basis for the extremely low activity of the PRL phosphatases toward artificial substrates in vitro. Thus, significant structural rearrangements would have to occur upon association of the PRL phosphatases with their physiological substrates and/or activators in order to enable efficient substrate turnover.

Unexpectedly, PRL-1 assembles into a trimer in the structures, burying 1400A2 of accessible surface area at each dimer interface. Trimerization creates a large, bipartite membrane-binding surface in which the exposed C-terminal basic residues could cooperate with the adjacent prenylation group to anchor PRL-1 on the acidic inner membrane. These structural observations are corroborated by mutational effects on PRL-1 trimerization and membrane localization.

Finally, native PRL-1 proteins are crystallized in the oxidized form in which a disulfide bond is formed between active site Cys104 and a neighboring residue Cys49, which would block both substrate binding and catalysis. Biochemical studies in solution and in the cell support a potential regulatory role of this intramolecular disulfide bond formation in response to reactive oxygen species such as H2O2.

A mouse model of conditional expression of oncogenic K-ras and deletion of p53: useful for ovarian cancer and lung cancer studies

Bailin Tan, Robert Bigsby Department of OB/GYN, Indiana University School of Medicine, Indianapolis, 46202

Oncogene K-ras activating mutations occur in 30% human cancers, in 25-50% of human lung adenocarcinomas and 58% mucinous ovarian tumors; on the other hand, tumor suppressor gene p53 inactivation was detected in 30-40% of ovarian carcinoma. Tumorigenesis frequently involves multi-gene mutations including proto-oncogenes and tumor suppressor genes. Therefore, we hypothesize that the combination of the oncogenic K-ras G12D and deletion of the tumor suppressor gene p53 will be sufficient to initiate the tumorigenic process. Using Cre/Loxp system, we have recently created a mouse model in which oncogenic K-ras G12D is conditionally expressed and p53 is conditionally deleted. Female mice (23) were treated by injecting an adenoviral vector expressing Cre-recombinase (Ade-Cre) under the ovarian bursal; the contralateral ovary was treated with empty vector. All ovaries treated with Ade-Cre developed malignant tumors within 5-8 weeks; no tumors developed in the control ovaries. In order to study the role of estrogen receptor in lung cancer development, we have tested lung tumorigenesis in this mouse model also. Male mice (12) were divided into two groups: with or without an estradiol (E2) capsule implanted s.c.. Female mice (8) were divided into two groups: with or without ovariectomy. Mice were examined at 10 weeks after intranasal instillation of Ade-Cre. Lung tumors were counted. In males all E2-treated animals developed lung tumors while 4 of 6 animals without E2 had tumors. E2 also increased the number and size of tumors in males. In female, all mice developed tumors. There was no difference in tumor number in ovariectomized and ovary-intact female mice. A key advantage of this animal model is the ability to conditionally induce tumorigenesis in adult tissues. These preliminary experiments indicate that this mouse model will be useful for studies of ovarian and lung cancers for studies of cancer development.

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EGFP Is a Useful Long-term Expression Tracer for Hematopoietic Stem Cells while DsRed Fluorescent Protein Is Not

Wen Tao[1,3,4], Barbara-Graham Evans[1,3,4], Scott Cooper[1,3,4], Kenneth Cornetta[1,2], Christopher B. Ballas[1,2], Giao Hangoc[1,3,4], and Hal E. Broxmeyer[1,3,4]
[1]Microbiology and Immunology, [2]Medical and Molecular Genetics, and [3]The Walther Oncology Center, Indiana University School of Medicine, Indianapolis, IN, USA; [4]The Walther Cancer Institute, Indianapolis, IN, USA.

In the post-genome era, precise functions of the vast majority of human and mouse genes and their interactions remain to be elucidated and defined. To assess the contributions of many different genes to hematopoiesis and to determine how they function in hematopoietic stem and progenitor cells, often requires introduction of the gene of interests or its derivative mutants into these cells along with a marker gene which is used to track the transduced cells over time. An ideal expression tracer should be easy to track and non-toxic to cells with minimal perturbation of cell metabolism. Although the enhanced green fluorescent protein (EGFP) has been widely used as an expression tracer and other fluorescent proteins were occasionally used in this capacity as well, their suitability for long-term marking of hematopoietic stem cells and their unintended side-effects on the functions of these cells have not been systematically investigated. In this study, we have performed a series of *in vitro* and *in vivo* experiments to evaluate whether two fluorescent proteins, EGFP and DsRed-Express which is an optimized variant of a red fluorescent protein from coral, are suitable for use as expression tracers in hematopoietic stem and progenitor cells. We first constructed a pair of MSCV based retroviral vectors with an identical backbone expressing either EGFP or DsRed-Express. These vectors were intended to be used for multicolor tracking of separate genes simultaneously and accurately in a single cell or mouse since the emission spectra of EGFP and DsRed-Express have minimal overlap. We used these vectors to transduce mouse mononuclear bone marrow cells, and the results demonstrated that the EGFP vector transduced green cells and DsRed-Express vector transduced red cells from single color or mixed dual color cell populations are clearly discerned by flow cytometry and fluorescent microscopy. Our results from *in vivo* competitive repopulation assay showed that under the experimental condition, mouse hematopoietic stem cells expressing EGFP alone are maintained nearly throughout the lifespan of the transplanted mice and appear to function normally. About 15 months after bone marrow transplantation, on average, 24% total peripheral white blood cells in recipient mice expressed EGFP. This initial donor population prior to injection contained 25.2% EGFP positive cells and all 5 mice assessed at 15 months were EGFP positive. Most EGFP transplanted mice lived at least 22 months and appeared normal at sacrifice. In contrast, the percentage of DsRed expressing donor cells transplanted either alone or mixed with EGFP expressing cells unexpectedly declined in recipient mice over time. By 3 months post-transplantation, the decrease of the percentage of DsRed expressing cells was dramatic. Therefore, EGFP itself has no detectable deteriorative effects on hematopoietic stem cells and is nearly an ideal long-term expression tracer for hematopoietic cells. However, the number of detectable DsRed expressing hematopoietic stem and progenitor cells, for reasons not yet known, decreases over time; therefore, DsRed fluorescent protein should not be used as a long-term tracer for these cells. This study also points out the importance of using correct expression tracers for accurately defining the functions of any genes.

Increased amount of intrinsic disorder in E6 and E7 oncoproteins from high risk HPVs

Vladimir N. Uversky[1-3], Ann Roman[4,5], A. Keith Dunker[1,2]

[1]Center for Computational Biology and Bioinformatics, Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, IN 46202, USA; [2]Molecular Kinetics, Inc., Indianapolis, IN 46268, USA; [3]Institute for Biological Instrumentation, Russian Academy of Sciences, 142290 Pushchino, Moscow Region, Russia; [4]Department of Microbiology and Immunology, and Walther Oncology Center, Indiana University School of Medicine; and [5]Walther Cancer Institute, Indianapolis, IN 46202, USA

It is recognized now that many functional proteins or their long segments are devoid of stable secondary and/or tertiary structure and exist instead as very dynamic ensembles of conformations. They are known by different names including natively unfolded, intrinsically disordered, intrinsically unstructured, rheomorphic, pliable, and different combination thereof. Many important functions and activities have been associated with these intrinsically disordered proteins (IDPs), including molecular recognition, signaling, and regulation. It is also believed that disorder of these proteins allows function to be readily modified through phosphorylation, acetylation, ubiquitination, hydroxylation, and proteolysis. Bioinformatics analysis revealed that IDPs comprise a large fraction of different proteomes. Furthermore, it is established that the intrinsic disorder is relatively abundant among cancer-related and other disease-related proteins and IDPs play a number of key roles in oncogenesis.

There are more than 100 different types of human papillomaviruses (HPVs), which are the causative agents of benign papillomas/warts, and cofactors in the development of carcinomas of the genital track, head and neck and epidermis. In respect to their association with cancer, HPVs are grouped into two classes, known as low- (e.g., HPV-6 and HPV-11) and high-risk (e.g., HPV-16 and HPV-18) types. The entire proteome of HPV includes eight major proteins: replication protein E1, replication and regulatory protein E2, proteins E4 and E5, transforming proteins E6 and E7 (that are known to function as oncoproteins in the high-risk HPVs), major capsid protein L1, and minor capsid protein L2. In order to understand whether intrinsic disorder plays a role in the oncogenic potential of different HPV types, we have performed a detailed bioinformatics analysis of proteomes of high-risk and low-risk HPVs with the major focus on E6 and E7 oncoproteins. The results of this analysis are consistent with the conclusion that high-risk HPVs are characterized by the increased amount of intrinsic disorder in transforming proteins E6 and E7.

Increased antitumor efficacy of dendritic cell vaccination through STAT3 deficiency in mouse

Zheng-Yu Wang, Akira Moh, Yongxue Yao, Wenjun Zhang, Cheong-Hee Chang, and Xin-Yuan Fu
IU Cancer Center; Department of Microbiology & Immunology, IU School of Medicine.

Dendritic cells (DCs) are bone marrow-derived antigen-presenting cells that play a critical role in T cell activation as well as T cell tolerance. In DC-based vaccination, the most common target of active immunotherapy strategies is the enhancement or modulation of the function of DCs. Here, using the mouse model with tissue-specific deletion of Stat3 during hematopoiesis, we observed that Stat3 deficiency enhanced the maturation status and overproduction of inflammatory cytokines by bone marrow-derived DCs from Stat3-deficient mice upon stimulation in vitro. In addition, STAT3 deficiency increased effect of STAT3-/- DC on CD4+ T cell proliferation and Th1 proliferation. These findings indicate that STAT3 may play an important role in negative regulation of DC maturation and activation. Furthermore, immunization of mice with STAT3-/- DC pulsed with OVA Ag more efficiently induced OVA-specific CTL of killing B16-OVA tumor cells *in vitro* and protecting against B16-OVA tumor subcutaneous growth *iv vivo*. Our studies raise the possibility that a combination with blocking DC STAT3 and pulsing DCs with Ag may improve the efficacy of DC-based cancer immunotherapies.

Potential Title: Toll-Like Receptor as a Marker and Functional Regulator of Hematopoietic Stem Cells

Abigail F. Weliver, Walther Oncology Center

Understanding regulation of hematopoietic stem cell (HSC) differentiation and self-renewal is crucial to furthering our understanding of hematopoiesis. Despite the biological and clinical importance of HSC, to date, factors regulating the activation and self-renewal of HSC remain largely unknown. Our initial purpose was to investigate the presence of Toll-like receptors (TLR) on primitive bone marrow cells. We discovered that a subset of the most primitive stem cells express TLR. We show here that c-Kit+ Sca-1+ Lineage– (KSL) bone marrow cells that express TLR2 and TLR4 (not previously described) phenotypically and functionally resemble previously described long-term repopulating HSC (LT-HSC). TLR+ KSL cells responded to exposure to peptidoglycan (PG) and lipopolysaccharide (LPS) with an increase in the number of lineage-negative primitive cells which form multiple-lineage colonies in vitro, and which successfully reconstitute all hematopoietic lineages upon transplantation into lethally irradiated hosts. Moreover, we describe in detail a novel ex vivo method of increasing the number of functional HSC utilizing the newly discovered presence of TLR on the bone marrow KSL, and investigate the role of TLR in stem cell fate determination. These data demonstrate a novel function of TLR in regulating hematopoiesis and linking innate immunology and hematology.

Effects of Activin on FSHβ promoter activity in Gonadotrope-Derived LβT2 Cell Lines

Crystal White and Christine Quirk

We have shown that the high mobility group (HMG) A family member, p8, plays a functional role in activation of the LHβ promoter in the gonadotrope-derived LβT2 cell line. *In vivo*, initiation of LHβ gene expression is delayed in gonadotropes of p8 knockout (-/-) mice compared to wild-type littermates. However, we believe p8 plays a more global role in gonadotrope cellular specification, impacting the gene expression of FSHβ as well as LHβ. Using stably-transfected LβT2 cell lines, in which p8 levels have been manipulated, we found the FSHβ promoter to be functional in control (C) LβT2 cells that express p8 endogenously as measured by luciferase reporter assay (2.25±0.13 fold higher than promoterless control). However, in LβT2 cells in which p8 expression has been stably knocked down (p8-KD-LβT2), the FSHβ promoter appears to be nonfunctional as luciferase levels are not different from the promoterless control and cannot be rescued upon treatment of cells with activin, indicating that p8 is necessary for activation of the FSHβ promoter in LβT2 cells. This data indicates that p8 is essential of the normal temporal expression of the LHβ and FSHβ genes both *in vitro* and *in vivo*.

IDENTIFICATION OF THE OLIGOMERIC STATUS AND THE OLIGOMERIZATION DOMAIN OF HUMAN MULTIDRUG TRANSPORTER ABCG2

JUNKANG XU, YANG LIU, YOUYUN YANG, AND JIAN-TING ZHANG

Multidrug resistance is a major problem in successful cancer chemotherapy. Human ABCG2 is one of the drug-efflux pumps that can actively transport a wide variety of anticancer drugs, and therefore, causes multidrug resistance. In the present work, the oligomeric status of ABCG2 was determined using gel electrophoresis, sucrose density gradient sedimentation, gel filtration chromatography, and immunoprecipitation. We found that the monomeric and homodimeric ABCG2s did not exist as major forms and that most human ABCG2s exist as homododecamer in plasma membranes, with tetramer as the minimum stable complex. The oligomerization domain of human ABCG2 was mapped to its transmembrane domain consisting of TM5-loop-TM6. This oligomerization domain, when expressed alone in HEK293 cells, also forms a homo-dodecamer. Furthermore, this domain can inhibit the drug efflux activity and drug resistance function of full-length ABCG2, likely by disrupting the formation of homo-oligomeric full-length ABCG2. These findings suggest that human ABCG2 likely exists and works as a homo-oligomer by interaction through its domain including TM5-loop-TM6 and that ABCG2 oligomerization may be used as a target for therapeutic development to circumvent ABCG2-mediated drug resistance.

The Rap1A GTPase plays multiple roles in mouse development and function

Jingliang Yan, Fang Li, Veerendra Munugalavadla, Hanying Chen, David Ingram, Reuben Kapur, Weinian Shou, Yu Li, and Lawrence Quilliam
Department of Biochemistry and Molecular Biology, Indiana University School of Medicine

The Ras related small GTPases, Rap1A and 1B, act as molecular switches to regulate many physiological processes. These include cell proliferation, adhesion, and migration. To further address the role of Rap1 and to determine the specific roles of the two closely related isoforms (encoded by two separate genes), we generated a Rap1A knock out mouse by disrupting the *rap*1A gene. Although the early progeny were viable, following backcross into theC57BL/6 mouse strain, F7 and later generations had a markedly decreased birth rate of homozygous pups. Examination of embryos revealed that most of the Rap1A-/- embryos had edema and heart defects. Furthermore, despite no postpartum lethality, adult mice, were prone to cardiomegaly. Rap1 is known to regulate endothelial cell-cell contact. To address if Rap1A might also contribute to angiogenesis, Matrigel plug and aortic ring assays were performed in the absence or presence of VEGF. Blood vessel formation was enhanced by Rap1A depletion in both assays. Rap1A null macrophages exhibited increased migration on several matrices that was attributable to their reduced adhesion. Although these cells exhibited reduced migration on plastic (that was not a result of altered adhesion), they had a similar fold chemotactic response to CXCL12 suggesting that despite a fundamental migratory defect, their chemotactic response was intact. These data suggest that Rap1A is required for many physiological events that include embryonic development, angiogenesis and leukocyte adhesion/migration. Additionally these findings demonstrate that the Rap1A and 1B isoforms serve unique/non-redundant biological function.

Binding Partners for the Apoptosis Regulatory Protein DAPK

Liguo Zhang, Emily Blue, and Patricia J. Gallagher Department of Cellular & Integrative Physiology, Indiana University School of Medicine, Indianapolis, IN USA 46202-5120

Death-associated protein (DAPK) is a Ca2+/CaM dependent, ser/thr protein kinase that has been suggested to be a tumor suppressor gene with multiple regulatory functions in apoptosis. This multi-domain, 160-kDa protein is comprised of different interaction domains which appear to mediate its functional participation in opposing pathways to result in elimination of premalignant cells via apoptosis or to promote cellular homoeostasis by its cytoprotective effects. A wealth of data has also shown that the gene for DAPK is frequently inactivated by aberrant promoter methylation (CpG) and this occurs in many cancer types, positioning this kinase for a role as a molecular marker for cancer prognosis.

Our recent studies have focused on identification of cellular proteins that interact with DAPK to mediate its activities. To this end we have identified several binding partners for DAPK. These proteins include: DAPK interacting protein (DIP1), TNFR1, RIP1, and HSP90. DIP1 is an E-3 ligase that has been shown to correspond to mindbomb (mib), which has a role in regulation of delta in the notch signaling pathway. DIP/mib also ubiquitinates DAPK to cause its degradation and thus provides an avenue for regulating cellular levels of DAPK. The receptor TNFR1 is a member of the death receptor family and mediates signaling for the inflammatory cytokine, TNF. Association of DAPK with TNFR1 appears to be constitutive. DAPK also interacts with RIP1, a death domain containing ser/thr kinase that participates in TNF signaling to activate NFkB for survival gene expression. Finally, results will show that HSP90 also interacts with DAPK. HSP90 is an abundant chaperone with numerous client proteins of which many are protein kinases. Inhibition of HSP90 with the tumor inhibitor, Geldanamycin, results in destabilization and degradation of DAPK. These multiple interactions suggest that DAPK is positioned as a convergence point regulating the balance between life and death signaling pathways.

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Phosphorylation contributes to the distinct form of RNA helicase A in drug-resistant vs drug-sensitive human leukemia cancer cells

Xiaoling Zhong and Ahmad R. Safa
Department of Pharmacology and Toxicology, IU Cancer Center,
Indiana University School of Medicine, Indianapolis, IN 46202

The development of multidrug resistance (MDR) phenotype in cancer is frequently associated with the overexpression of the *MDR1* gene product P-glycoprotein (P-gp). We have previously identified a multiprotein complex that is bound to the *MDR1* gene promoter and analyzed one component of the complex, which is RNA helicase A (RHA) (J. Biol. Chem. 279:17134-17141; 2004). Overexpression of RHA enhanced activity of the *MDR1* promoter and increased P-gp level in the drug-resistant human leukemia HL-60 cells but not in the drug-sensitive counterpart. We have also found that distinct forms of RHA exist in these two cell lines, with the higher molecular mass present only in the drug-resistant cells. In the present study, we investigated whether the mass difference is due to phosphorylation of RHA. Western Blotting analysis of the phosphatase treated nuclear proteins obtained from the drug-resistant cell showed that the slower migrating RHA was converted to a faster one, which has an electrophoretic mobility similar to that from the drug-sensitive cells. Phosphatase treatment of the RHA immunoprecipitates resulted in a dephosphorylated RHA band as revealed by phosphoprotein staining. Because protein phosphorylation requires the action of protein kinase and our previous analysis of the multiprotein complex by mass spectrometry revealed that DNA-PKcs is a component, we immunoprecipitated RHA from the drug-resistant cells and blotted DNA-PKcs with DNA-PKcs antibody. The result showed an association of DNA-PKcs with RHA, while DNA-PKcs in the drug-sensitive cells was undetectable. It is known that the active form of DNA-PKcs is dephosphorylated and protein phosphatase PP2A activates the activity of the kinase. To further characterize RHA phosphorylation *in vivo*, we treated the drug-resistant cells with okadaic acid, a PP2A inhibitor. The treatment resulted in the band shift from the higher mass to the lower one as evidenced by Western Blotting. These results demonstrate that the endogenous RHA in drug-resistant HL-60 cells is phosphorylated. Study with siRNA targeting DNA-PKcs to further determine whether DNA-PKcs silencing leads to RHA dephosphorylation *in vivo* is in progress. To investigate the functional consequences of RHA phosphorylation, we expressed the full-length His-tagged RHA in insect cells with the baculovirus expression system. The baculovirus-RHA is also a phosphoprotein as analyzed by phosphoprotein staining. We predict that phosphorylation state of RHA affects its regulation of the *MDR1* promoter. Collectively, RHA is phosphorylated in the drug-resistant HL-60 cancer cells and the phosphorylation may contribute to the overexpression of P-gp and the development of drug resistance (supported by RO1 CA 90878 grant to ARS).

Identification of Functional Genetic Variants of the Indoleamine 2,3 Dioxygenase Gene

Arefayene, M., Mamidipalli, S., Philips, S., Cao, D., Flockhart, D., Wilkes, D., Skaar, T.
Indiana University School of Medicine
Indoleamine 2, 3- dioxygenase (IDO) is a cytosolic enzyme that catalyses the oxidative metabolism of L- tryptophan to kynurenine. By regulating local concentrations of tryptophan and kynurenine, IDO regulates local function immune cells. IDO expression is highly inducible by cytokines in multiple cell types, including some cancer cell lines. Previous studies have shown that IDO is expressed in many types of human tumors. In preclinical studies, inhibiting IDO activity synergizes with other therapeutic drugs and can also affect tumor growth by itself. In addition to the direct effects on tumors, elevated IDO activity in response to interferon therapy can also cause substantial side effects, such as depression, in cancer patients. There is a large amount of interindividual variation in the IDO expression in cancer patients, which may affect therapeutic responses and susceptibility to drug side effects. We hypothesized that there are genetic variants in the IDO gene that contribute to the interindividual variability in IDO activity. The goal of this study was to identify germline genetic variants in the IDO gene and test their functionality, *in vitro*.
We resequenced the exons and intron-exon borders of the IDO gene in 96 DNA samples from the DNA diversity panels from the Coriell DNA Repository. We identified 15 single nucleotide polymorphisms and one 9 bp deletion. Eight of these SNPs are novel findings and 7 confirmed previously identified SNPs. Two of the newly identified SNPs were nonsynomous, one in exon 1 and one in exon 3. The 9 bp deletion variant coded for a 3 amino acid deletion in exon 7 in the cDNA. We obtained the wild-type IDO cDNA in a mammalian expression vector and created the exon 1 and exon 3 variant cDNAs by site directed mutagenesis. We expressed the wild-type and variant cDNAs in COS-7 cells and determined enzyme activity by measuring tryptophan and kynurenine concentrations in the cultured media 48 hours after transient transfection. The exon 3 variant cDNA had >90% reduction in activity compared to the wild-type and exon 1 variants.
We conclude that the nonsynonomous SNP in exon 3 of the INDO gene is a low tryptophan metabolic activity allele. This may have important implications for understanding the local immune response in tumors.

In vivo selection of murine long-term repopulating cells transduced with a foamy virus vector that expresses MGMTP140K.

Shanbao Cai, Aaron Ernstberger, Scott Goebel, Helmut Hanenberg, and Karen E. Pollok.

Recombinant foamy virus vectors transduce noncycling and cycling cells, are stable episomally, and integrate into the host genome during cell division. Due to the cytoplasmic stability of this vector, a substantial lag period between transduction and cell division required for provirus integration is possible. Therefore, in transplantation studies that use minimally stimulated hematopoietic stem and progenitor cells (HSC), integration of the foamy virus vector in HSC may occur once HSC divide post-transplantation. We used a foamy virus vector, MD9-P140K-EGFP, that co-expresses a mutant form of O^6-methylguanine DNA methyltransferase (MGMTP140K) and the enhanced green fluorescent protein (EGFP) to test the hypothesis that HSC could be transduced with a foamy virus vector and selected in vivo by alkylator-based chemotherapy. We also compared foamy virus transduction and selection to our previously optimized strategy using an oncoretrovirus vector to express MGMTP140K (SF1-P140K-IRES-EGFP). Lineage-depleted bone marrow (BM) from C57BL/6 mice was transduced for 10-16 hours with the foamy virus vector or following a 2-day prestimulation with the oncoretrovirus vector. Data presented are from three primary transplant experiments analyzed over 6 months and one secondary transplantation experiment analyzed for 6 months. The bulk transduction efficiency using the foamy virus vector ranged from 12-25% and the CFU transduction efficiency was 55-57%. Transductions with the oncoretrovirus vectors resulted in similar bulk and CFU transduction efficiencies (55-60%). Similar numbers of progenitor colonies (oncoretrovirus vs. foamy virus) were observed. MGMT activity in pooled progenitor colonies was 10-fold higher in $EGFP^+$ versus $EGFP^-$ colonies. Although similar levels of CFU were transduced using the two vector systems, significantly different levels of in vivo selection were obtained in primary recipient mice. Consistent with previous studies, selection of oncoretroviral vector-transduced cells resulted in high and sustained levels of $EGFP^+$ cells in the PB and BM in primary and secondary recipient mice (80-99% $EGFP^+$ with 2-3 cycles of 6BG/BCNU). For primary transplants using cells transduced with the foamy virus vector, EGFP expression in the PB peaked at 3 months post-treatment (26.2+/-4.0%) which represented a 4-6 fold increase compared to vehicle-treated mice. However, by 6 months EGFP expression dropped by 3-fold (9+/-1%). Western analysis of MGMT protein levels found in the BM at 6 months post-transplantation however, showed a 2-3-fold increase in expression. In secondary reconstitution experiments, flow cytometry and Western analysis of MGMT expression indicated that EGFP expression in foamy virus-transduced HSC no longer correlated with MGMT expression. In fact MGMT expression levels following drug treatment were similar to those found in secondary recipient mice transplanted with oncoretroviral vector-transduced cells, suggesting that stem cells expressing MGMT were selected over time. These data demonstrate that although foamy virus transduction is not as efficient as the more commonly studied oncoretrovirus transduction strategy, a simple overnight protocol can be used to transduce minimally stimulated HSC with a foamy virus vector. These cells can be selected in vivo, can reconstitute mice for up to one year, and can maintain high levels of MGMT expression.

Hyper-IgM Syndrome of Uncertain Molecular Character

Cavalier, Maryellen[2]; Pradhan, Kamnesh[2]; Nelson, Robert P.[1].

Internal Medicine, Division of Hematology/Oncology, Hematology malignancy Program and Stem Cell Transplantation, Indiana University School of Medicine, Indianapolis, IN, USA.
2. Pediatrics, Division of Pediatric Hematology/Oncology, Indiana University School of Medicine, Indianapolis, IN, USA.

A 16-month-old female presented with a history of fourteen episodes of otitis media; disseminated herpes zoster following immunization; pancytopenia; and massive splenomegaly. Family history was remarkable for mother's (age 30) having CVID and splenomegaly. Laboratory evaluation revealed the following: serum IgM, 1290mg/dl; IgG < 33mg/dl, IgE, < 1mg/dl, IgA, < 1 mg/dl. Peripheral blood immunophenotyping was remarkable for the following: CD3+, 75%; CD4+, 71%; CD8+, 4%; CD19+, 9%, CD16+/CD56-, 16%. Antibody responses to protein antigens were absent; random titers to several strains of Streptococcus pneumoniae were minimal. T cell proliferation to mitogens and antigens was profoundly decreased. Mutational analyses of CD40, CD40 ligand, activation-induced cytidine deaminase (AID) and uracil-DNA glycosylase (UNG) gene sequences were normal. Adenosine deaminase and purine nucleoside phosphorylase were normal. Mutational analysis of interleukin-2 gamma receptor was normal.

At 20 months of age, the child developed fevers, marked retroperitoneal and inguinal lymphadenopathy, worsening splenomegaly, and ground glass pulmonary opacities. IgM was 3800mg/dL. Histopathological analysis of lymphatic tissue, liver, lung, and bone marrow revealed atypical lymphoproliferation without evidence of clonality by T and B-cell gene rearrangements. Lung biopsy AFB and fungal stains were negative, but cultures grew Mycobacterium mucigens. The patient's life-threatening hypersplenism improved with the administration one dose of cyclophosphamide and vincristine. Concomitant anti-tuberculous therapy was initiated.

This case describes clinical characteristics of a novel form of hyper-IgM: high IgM with absence of other isotype production, severe T cell dysfunction, polyclonal lymphoproliferation, opportunistic mycobacterial infection and mother with clinical phenotype consistent with common variable hypogammaglobulinemia

Title: Adoptive Transfer of *Nf1+/-* Bone Marrow Is Sufficient For Neurofibroma Progression in Krox20;*Nf1*flox/floxMice.

Shi Chen, Feng-Chun Yang, David A Ingram, Xiaohong Li, Trent Morgan, Jin Yuan, Yan Li, Gary D Hutchins, Luis Parada and D. Wade Clapp. Department of Pediatrics, Indiana University, Indianapolis, IN, United States; Center for Developmental Biology, U. Texas SW, Dallas, TX, United States; Department of Radiology, IU, Indiana University School of Medicine, Indianapolis, IN 46202, Indianapolis

Background: Mutations in the *NF1* tumor suppressor gene cause neurofibromatosis type 1 (NF1). Neurofibromas, the hallmark of NF1, are complex tumors characterized by tumorigenic Schwann cells (SCs), neoangiogenesis, fibrosis, and degranulating mast cells (MCs). Our lab provided the first genetic evidence that haploinsufficiency of *Nf1* alters ras activity and cell fates in MCs and identified a mechanism underlying the recruitment of MCs to tumorigenic SCs. Other studies found that nullizygosity of *Nf1* in SCs of conditional knockout mice (Krox20;*Nf1*flox/flox) was necessary but not sufficient for neurofibroma formation and haploinsufficiency of *Nf1* in lineages within the tumor microenvironment was required for neurofibroma progression.
Objective: The aim of this study is to determine whether haploinsufficiency of *Nf1* in the hematopoietic system alone is sufficient for the development of neurofibromas in Krox20;*Nf1*flox/floxmice.
Design/Methods: *Nf1+/-* or wildtype (WT) GFP positive bone marrow (BM) was transplanted into irradiated Krox20;*Nf1*flox/flox mice and cohorts were followed prospectively using PET/CT imaging. The spinal cord, peripheral nerves and dorsal root tumors were analyzed histologically. Immunohistochemical staining was performed to evaluate whether tumors contained the typical cellular features of neurofibromas including SC invasion, increased MCs, fibrosis, and neoangiogenesis.
Results: Mice transplanted with *Nf1+/-* but not WT BM developed progressive enlargement of the trigeminal nerve, dorsal root ganglia, peripheral nerves, and motor paralysis similar to Krox20;*Nf1*flox/flox mice that are haploinsufficient at *Nf1* in all lineages of the tumor microenvironment. Postmortem analysis revealed that Krox20;*Nf1*flox/flox transplanted with *Nf1+/-* BM had cellular neurofibromas containing SCs, fibroblasts, blood vessels and MCs. Mice transplanted with WT BM did not develop neurofibromas.
Conclusions: These studies indicate that haploinsufficiency of Nf1 in the the hematopoietic system is sufficient for neurofibroma formation in Krox20;*Nf1*flox/flox- mice and implicate a role for targeting molecular therapies to the hematopoietic system to treat or prevent neurofibroma progression.

Significant contribution of CYP3A5 to the metabolism of vincristine in vitro

Vincristine chemotherapy in the treatment of acute lymphoblastic leukemia is characterized by unpredictable neurotoxicity and interracial differences in efficacy. Studies that evaluated the pharmacokinetics of vincristine have shown a 20-fold range in drug exposure. In vitro and in vivo data support a dominant role for CYP3A enzymes in the elimination of vincristine. Consequently, genetic polymorphisms in CYP expression may contribute to the interindividual variability in clinical response. Despite the long history of clinical use, the primary pathways of vincristine metabolism have not been defined. In the present study, the major P450 mediated metabolites were identified and the roles of CYP3A4 and CYP3A5 were defined in a recombinant system. In addition, the effect of CYP3A5 genotype on the metabolism of vincristine was evaluated in human liver microsomes. CYP3A4 and CYP3A5 were the only CYPs to support substantial loss of parent drug and formation of the previously unidentified, major metabolite (M1). In contrast to most CYP3A biotransformations, the oxidation of vincristine was considerably more efficient with CYP3A5 than CYP3A4 (9 to 14-fold higher intrinsic clearance for CYP3A5). Data from human liver microsomes indicated that the majority of the metabolism was mediated by CYP3A5 in individuals heterozygous for the *CYP3A5*1* allele. The intrinsic clearance of vincristine was on average 3-fold higher for *CYP3A5*1* heterozygous expressors compared to *CYP3A5*3/*3* individuals. The large variability in intrinsic clearance for *CYP3A5*3/*3* individuals was correlated to 6B-testosterone activity, a measure of CYP3A4 activity. Correcting for CYP3A4 activity, the contribution of CYP3A5 to the metabolism of *CYP3A5*1* heterozygous expressors was between 62-85%. We conclude that common genetic polymorphisms in CYP3A5 expression may contribute to the interindividual variability in the systemic elimination of vincristine.

Jennifer Dennison, PhD student in Pharmacology.

Engraftment Kinetics after Truly Nonmyeloablative Matched Related and Unrelated Allogeneic Transplantation

Finke, Teresa B.[1]; Nelson, Robert P.[2]; Dlouhy, Stephen R.[5]; Abonour, Rafat[2]; Robertson, Michael J.[2]; Cornetta, Kenneth[3]; Czader, Magdalena B.[4].

1. Department of Medicine and Pediatrics, Indiana University School of Medicine, Indianapolis, IN, USA. 2. Internal Medicine, Division of Hematology/Oncology, Hematology malignancy Program and Stem Cell Transplantation, Indiana University School of Medicine, Indianapolis, IN, USA. 3. Department of Medical and Molecular Genetics; Department of Pathology and Laboratory Medicine, Indiana University School of Medicine, Indianapolis, IN, USA. 4. Pathology, Division of Hematopathology, Indiana University School of Medicine, Indianapolis, IN, USA. 5. Department of Medical and Molecular Genetics, Indiana University School of Medicine, Indianapolis, IN, USA.

Forty-nine consecutive patients with hematological malignancies (median age, 55 years) received PBMCs from a matched related (MRD) or unrelated (MUD) donor following immunosuppressive dosages of cyclophosphamide and fludarabine. Cyclosporine +/- mycophenolate mofetil (MMF) were given as GVHD prophylaxis. Total cell dosages infused on Day 0 ranged from 0.8 to 12.6 X 106 CD34+ cells/kg recipient body weight (mean, 5.1 x 106 cells/kg). Donor engraftment was defined as an ANC > 500/mm3 and >5% donor
peripheral blood mononuclear cell (unfractionated) chimerism at day +30. Pre- and post-transplant bone marrow aspirates and/or biopsies were examined from 31 patients (median age, 56 years) and analyzed histologically for cellularity, differential and fibrosis.

All patients engrafted without growth factor support and with similar chimerism patterns after related (n=26) or unrelated (n=23) transplantation. MRD and MUD recipients reached ANCs > 500/mm3 at median times of 15 and 12 days, respectively. 84% of MRD and 91% of MUD recipients achieved ≥ 50% donor engraftment by day +30, and 80% of each group were 100% donor at 6 months. Median time to ANC > 2000/mm3 for those without or with MMF were 17.5 days and 18 days, respectively (p=0.75). Nine subjects failed to achieve > 80% donor chimerism at day +100 for the following reasons: disease progression prior to donor chimerism (3);disease progression after 100% donor (2); death from infection prior to 100% donor chimerism (1); conversion to 100% donor after stopping immunosuppression(2); and, achievement of prolonged mixed chimerism, transfusion-free MDS for 14 months and then transformation to AML(1).

These findings confirm a high engraftment rate in recipients of truly NMAT. Recipients of MRD and MUD transplants in this moderately sized cohort experienced similar engraftment/donor chimerism patterns.

Enhancement of ovarian cancer treatment via manipulation of the base excision repair pathway after temozolomide treatment

Melissa L. Fishel, Ying He, Shanbao Cai, Karen E. Pollok, and Mark R. Kelley
Dept. of Pediatrics, Section of Hematology/Oncology, Herman B Wells Center for Pediatric Research, Indiana University School of Medicine, 1044 W Walnut Ave. Indianapolis, IN 46202

Ovarian cancer is the fourth-leading cause of cancer death in women, and the incidence of this dreaded cancer continues to increase. Although women are encouraged to undergo annual physical examinations and pap smears, there are currently no available screening tests for the early detection of ovarian cancer. Consequently, three-fourths of women with ovarian cancer present with advanced disease, and the majority of these women will ultimately succumb. Over the past three decades, improved surgical techniques and the identification of new chemotherapy agents such as cisplatin and its derivatives, and more recently paclitaxel, have yielded modest improvements in survival. Nevertheless, virtually all women who die from ovarian cancer have metastasis that are resistant to platinum agents and other drugs. Future reductions in ovarian cancer mortality can only be obtained through the identification and implementation of effective screening tests for apparently-healthy women, and through the identification and implementation of better treatments.

Our efforts are aimed at improving the treatment of women with ovarian cancer. To accomplish this, we are investigating the modulation of prominent DNA damaging agent: temozolomide (TMZ). Methoxyamine (MX), a DNA base excision repair (BER) inhibitor, potentiates TMZ-induced cytotoxicity by blocking downstream members of the BER pathway. Although clinical trials in ovarian cancer to determine TMZ single-agent efficacy are in development, it is effective in other types of cancer such as astrocytoma, malignant melanoma, and glioblastoma multiforme. The combination has potential for second-line therapy for patients who have failed standard platinum plus paclitaxel chemotherapy. Our data demonstrates that we can effectively modulate the activity of chemotherapeutic agent, TMZ via modulator MX, in three ovarian cancer cell lines. MX is capable of enhancing the cytotoxicity and level of DNA damage after TMZ treatment in SKOV-3x, Ovcar-3 and IGROV-1 cells. In addition to cell line studies, an in vivo model using NOD/SCID mice, demonstrates the efficacy of the combination of TMZ and MX in xenograft studies. In efforts to further manipulate the BER pathway and enhance cell killing, we have used adenoviral overexpression of N-methylpurine DNA glycosylase (MPG) in addition to MX + TMZ treatment in ovarian cancer cells. Our results show that MPG-overexpressing IGROV-1 cells are significantly more sensitive to the clinical chemotherapeutic TMZ as assayed by cytotoxicity, apoptosis, and levels of DNA damage. These studies may directly identify a more effective way of treating ovarian cancer with chemotherapy, and indirectly lead us to other potential targets to manipulate to better treat patients with ovarian cancer. Effective modulation of chemotherapeutic agents is necessary both to improve efficacy and to attenuate toxicity in order to effectively treat ovarian tumors where cisplatin is not as effective.

Effects of Telomerase Inhibition in MDA-MB-231 Breast Cancer Cells

Erin Goldblatt[1*], Amelia Hochreiter[1], Hongling Xiao[1], and Brittney-Shea Herbert[1, 2]
[1]Department of Medical and Molecular Genetics, and [2]Indiana University Cancer Center, Indiana University School of Medicine, Indianapolis, Indiana

Introduction: The maintenance of functional telomeres at chromosome ends is essential for cell survival and preventing critically short telomeres from being fused together, inducing genomic instability, or being recognized as damaged DNA needing repair. The telomerase reverse transcriptase complex has been shown to maintain telomere length. Telomerase is inactive or undetectable in most somatic cells, but has been shown to be active in over 90% of cancers. Therefore, inhibition of telomerase activity has been proposed as a potential cancer therapy. Several telomerase inhibitors have been designed and studied in cancer cells. For example, GRN163L has been shown to be a novel, potent inhibitor of telomerase activity in different cancer cell lines. The purpose of this study was to examine the effects of telomerase inhibition on MDA-MB-231 breast cancer cells.

Methods: MDA-MB-231 cells were treated with various doses of the telomerase inhibitor GRN163L. Telomerase activity, telomere length, viability, colony formation, cell cycle analysis, and *in vivo* tumor growth were measured. Combination studies were also conducted with irradiation, doxorubicin, and etoposide to examine the ability of telomerase inhibitors to sensitize cells to other cancer therapies.

Results: GRN163L inhibited telomerase activity in a dose dependent fashion, as well as decreased telomere length. Treatment with GRN163L did not induce immediate toxicity to MDA-MB-231 cells. However, GRN163L significantly reduced colony formation and *in vivo* tumor growth. Combining GRN163L with irradiation, doxorubicin, or etoposide also resulted in decreased cell growth.

Conclusion: The effects on cell survival and tumor growth suggest that the maintenance of telomeres is crucial for the metastatic potential and chemo- and radio-sensitivity of these breast cancer cells. These results suggest that the telomerase inhibitor GRN163L can be an effective breast cancer therapy. Future studies include investigation into the effects of GRN163L on cancer stem cells.

*975 W. Walnut St, IB 239, Department of Medical and Molecular Genetics, Indiana University School of Medicine, Indianapolis, Indiana 46202
egoldbla@iupui.edu

Antitumor Activity of Ad-IU-2, a Prostate-Specific Replication-Competent Adenovirus Encoding Apoptosis Inducer, TRAIL.

Juan Antonio Jiménez, Xiong Li, Kyung Hee Bae, Yan-Ping Zhang, Chinghai Kao, and Thomas A. Gardner

Indiana University School of Medicine; Departments of Urology, Microbiology and Immunology; Walther Oncology Center.

In 2005, it is estimated that prostate cancer will account for the most new cancer diagnoses, aside from skin cancer, at 232,090 men in the United States and will be the second most common cause of cancer deaths at 30,350 men. Currently, 25% of men treated for localized prostate cancer will experience biochemical disease recurrence within ten years of treatment, and all recurrent tumors become refractory to hormone therapy. Here, we investigate the antitumor effect of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) delivered by Ad-IU-2, a prostate-specific replication-competent adenovirus (PSRCA), against androgen-independent prostate cancer. Ad-IU-2 was made by placing adenoviral *E1a* and *E4* genes under the control of the bidirectional chimeric enhancer prostate-specific enhancing sequence (PSES) upstream from the right ITR as well as *E1b* and membrane-bound TRAIL cDNA under the control of PSES downstream of the left ITR. Expression of early adenoviral genes and TRAIL was limited to prostate-specific antigen (PSA) and prostate-specific membrane antigen (PSMA)-positive cells. Furthermore, Ad-IU-2 induced apoptosis specifically in PSA/PSMA-positive cells above that induced by a PSRCA. Likewise, the tumor cell killing activity was two-fold greater than that of a PSRCA. The growth of subcutaneous androgen-independent CWR22rv tumors in the flanks of athymic nude mice was inhibited following intralesional injection of Ad-IU-2. TRAIL-mediated bystander killing of adjacent PSA/PSMA-negative PC-3 cells was observed and augments the killing power of a PSRCA. Ad-IU-2 is an effective molecular therapeutic for androgen-independent prostate cancer due to its strong tissue-specificity and powerful TRAIL-mediated antitumor activity and bystander killing effect.

Submitting Author: Juan A. Jiménez jjimenez@iupui.edu

Drug-Mediated Uptake of Fluorescent Nanocrystals by Colon Cancer Cells*

**Giselle M. Knudsen[1] , Roman V. Rozhkov[1] , Jennifer Sturgis[2] , Alexander E. Ribbe[4] ,* *J. Paul Robinson[2],3 , V. Jo Davisson*^1,3

^1 Medicinal Chemistry & Molecular Pharmacology, Purdue University, 575 Stadium Mall Dr., West Lafayette, IN, 47907, ^2 Basic Medical Sciences and the ^3 Bindley Bioscience Center, Purdue University, 201 South University Dr., West Lafayette, IN, 47907, ^4 Purdue Laboratory of Chemical Nanotechnology, Purdue University, 560 Oval Dr. BRWN, West Lafayette, IN 47907.

***Abstract*

A polyacrylate coated CdSe/ZnS nanocrystal (NC) has been synthesized that allows for well controlled surface conjugation with derivatives of the ansamycin antitumor agent geldanamycin. Inclusion of the pharmacologic agent on the surface enabled cellular uptake of the polymer coated nanoparticles by HCT116 colon cancer line. The efficiency of cellular uptake was correlated with the ratio of surface conjugated geldanamycin derivative to polymer coated nanoparticle. This is the first example of dose-responsive drug-mediated uptake of nanoparticles by living cells.

Combination Therapy of Androgen-Independent Prostate Cancer Using A Prostate Restricted Replicative Adenovirus and A Replication-Defective Adenovirus Encoding Human Endostatin-Angiostatin Fusion Gene

Xiong Li,[1,5] Sudhanshu P. Raikwar,[1,5] You-Hong Liu,[1,5] Sang-Jin Lee,[1,5] Yan-Ping Zhang,[1,5] Shaobo Zhang,[4] Liang Cheng,[1,4] Sang-Don Lee,[1,5] Beth Elisa Juliar,[6] Thomas A. Gardner,[1,2,5] Meei-Huey Jeng,[2,3,5] and Chinghai Kao[1,2,5]
Author's Affiliations: Departments of [1]Urology, [2]Microbiology and Immunology, [3]Medicine and [4]Pathology, [5]Walther Oncology Center, [6]Division of Biostatistics, Indiana University School of Medicine, Indianapolis, IN

Although prostate restricted replicative adenovirus (PRRA) has exhibited significant anti-tumor efficacy in pre-clinical studies, it is necessary to develop more potent adenoviruses for prostate cancer (PCa) gene therapy. We evaluated the synergistic killing effect of PRRA and AdEndoAngio, a replication-defective adenovirus expressing the endostatin-angiostatin fusion protein (EndoAngio). When co- administered with AdEndoAngio, PRRA significantly elevated EndoAngio expression, suggesting that AdEndoAngio co-replicates with PRRA. Conditioned medium (CM) from PCa cells infected by PRRA plus AdEndoAngio inhibited the growth, tubular network formation and migration of human umbilical vein endothelial cells better than CM from PCa cells infected by AdEndoAngio alone. Furthermore, the *in vivo* animal studies demonstrated that the co-administration of PRRA plus AdEndoAngio resulted in complete regression of 7 out of 8 treated androgen independent (AI) CWR22rv tumors, with a tumor nodule maintaining a small size for 14 weeks. The residual single tumor exhibited extreme pathological features together with more endostatin-reactive antibody-labeled tumor cells and fewer CD31 reactive antibody-labeled capillaries than the AdEndoAngio-treated tumors. These results show that a combination therapy utilizing PRRA together with antiangiogenic therapy has more potent anti-tumor effects and advantages than single PRRA and deserves more extensive investigation.

Key words: PSA, PSMA, PSES, antiangiogenesis, gene therapy, prostate cancer, oncolytic adenovirus, endostatin, angiostatin

Signaling studies of PRL-3, a phosphatase associated with cancer metastasis

Fubo Liang, Wei-qing Wang, Jiao Liang, Jinpeng Sun, Zhong-Yin Zhang
Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, IUPUI, 46224

Abstract Colorectal cancer (CRC) is the second leading cause of cancer-related death in the United States, and metastasis of CRC to liver and other organs is often responsible for the incidence of mortality. Recently, by comparing the global gene expression profile of metastatic CRC with that of primary tumors, normal colorectal epithelium, PRL-3 is identified as the only gene that is highly expressed in all metastases examined. In contrast, little or no PRL-3 expression is observed in normal colon or primary cancers. Ectopic expression of PRL-3 in a variety of cell lines leads to transformation and experimental metastasis, and the catalytic activity of PRL-3 is required for the observed effects. These findings suggest that PRL-3 could serve not only as a prognostic marker but also a therapeutic target for CRC metastasis treatment. In this work, we examined the effects of the expression of wild type as well as catalytically mutant PRL-3 (C104S) in HEK293 cells. We observed that expression of PRL-3 leads to higher proliferation rate, more motile cell morphology, higher migration rate and cell growth in soft agar, suggesting PRL-3 could be a causative factor for both tumorigenesis and metastasis. Biochemical studies showed that the CSK-Src-Erk pathway is activated, which is partially responsible for the increased migration capacity of PRL-3 cells. PRL-3 expression leads to decreased CSK protein synthesis without affecting its protein degradation as well as mRNA transcription rate. These results indicate that PRL-3 affects multiple cellular physiologies, which functions upstream of CSK.

Engraftment and Clearance of Paroxysmal Nocturnal Hemoglobinuria (PNH) Cells after Nonmyelablative Unrelated Transplant in a Highly-Transfused Patient

Nelson, Robert P.[1]; Robertson, Michael J.[1]; Schwartz, Jennifer E.[1]; Vance, Gail H.[2]; Ward, Kelly E.[1]; Griep, John A.[3]; Czader, Magdalena[3]; Abonour, Rafat [1].

1. Internal Medicine, Division of Hematology/Oncology, Hematology Malignancy Program and Stem Cell Transplantation, Indianapolis, IN, USA. 2. Department of Medical and Molecular Genetics, Indiana University School of Medicine, Indianapolis, IN, USA. 3. Departments of Pathology and Laboratory Medicine , Indiana University School of Medicine, Indianapolis, IN, USA.

A 37-year-old was diagnosed with aplastic anemia at the age of 19 and was treated at various times with erythropoietin, prednisone, and danacrine. She received over 300 transfusions over 17 years. Pre-transplant evaluation revealed loss of glycosylphosphatidylinositol-anchored antigens CD55 and CD59 consistent with PNH. She was allosensitized to blood group antigens E, c, K, Cw. Alveolar lavage done to evaluate chronic had bilateral ground glass infitrates showed only hemosiderin-laden macrophages. She received 2.2 X 106 CD34+ and 2.0 x 108 CD3+ cells/kg of 10/10 HLA- and ABO-matched unrelated donor PBMCs following immunosuppressive dosages of cyclophosphamide and fludarabine. Cyclosporine (CSA) and mycophenolate mofetil were used for GVHD prophylaxis.
She experience no mucositis, engraftment syndrome day +8 that resolved with moderate-dose steroids, sustained engraftment by day +12 and posterior reversible encephalopathy syndrome, which resolved temporally with switching CSA to tacrolimus. Flow cytometric analysis of CD55 and CD59 showed no evidence of PNH by day +25 and she was discharged on Day +26. Peripheral blood cell chimerism was 100% donor on day +30. With 100% PBMC donor cell chimerism at day +106, bone marrow morphology revealed trilineage hematopoiesis, mild erythroid and mast cell hyperplasia. Immunoreconstitution thus far reveals the following on Day +106: Serum IgG, 463mg/dL; WBC, 4,400; ALC, 880/mm 3; CD2, 92%; CD3, 80%; CD4, 29%; CD8, 50%; CD16, 16%, Cd19, 1%. She is currently day +126, transfusion-independent without GVHD. This report documents sustained early engraftment and elimination of the malignant clone in a highly allo-sensitized adult across unrelated minor histocompatability barriers without radiation- or ATG-containing conditioning.

Graft-versus-Host Disease (GVHD) after Nonmyeloablative Transplantation

Nelson, Robert P.[1]; Robertson, Michael J.[1]; Schwartz, Jennifer E.[1]; Billings, Steven D.[2]; Abonour, Rafat[1].

1. Internal Medicine, Division of Hematology/Oncology, Hematology Malignancy Program and Stem Cell Transplantation, Indiana University School of Medicine, Indianapolis, IN, USA.
   2. Departments of Pathology and Laboratory Medicine and Dermatology, Indiana University School of Medicine, Indianapolis, IN, USA.

Forty-nine consecutive patients with hematological malignancies (median age, 55 years) received HLA-matched PBMCs following immunosuppressive dosages of cyclophosphamide and fludarabine. GVHD prophylaxis included cyclosporine +/-mycophenolate mofetil (MMF). Patients were followed until death or at least 7 months (range, 25-1675 days; median 413 days). Overall rates of aGVHD and grade 3 or 4 aGVHD were 65.3% (32/49) and 28.6% (14/49); no patients experienced antecedent mucositis and rates were equivelent for MRD (n=26) and MUD (n=23) recipients. Median time to onset was 34 days. “Late onset” acute GVHD was diagnosed in 10 patients at a mean of 109.8 days, 8 of whom had concomitant histological features of chronic lichenoid GVHD (see Figure). Overall rates chronic GVHD (cGVHD) were 69.8% (57.2% extensive), diagnosed at a median of day +168; 50% (13/26) in the MRD and 69% (11/16) MUD groups, respectively (p=0.344). Extensive cGVHD was associated with the presence of grade 3 or 4 aGVHD Fourteen of 24 (58%) of survivors are living with extensive cGVHD, 11 (78.5%) of whom are tolerating reductions in immunosuppressive therapy. Two (14.3%) are requiring pulmonary specialty care for symptomatic bronchiolitis obliterans; no patients have experienced progressive sclerotic dermal disease, malabsorption or chronic liver disease.
Clinical features GVHD that differ from that typically observed after conventional transplantation include time of onset and no requirement for mucositis as an initiator. We observed no difference in GVHD incidence in those who did and did not receive MMF. cGVHD may resolve in some patients, suggesting tolerance occurs over time in some older patients, even with unrelated donors.

Outcomes after Nonablative Cyclophosphamide/Fludarabine Conditioning and Related/Unrelated Allotransplantation for Hematological Malignancies

Nelson, Robert P.[1]; Robertson, Michael J.[1]; Cripe, Larry D.[1]; Hromas, Robert[1]; Yu, Menggang[2]; Schwartz, Jennifer E.[1]; Vance, Gail H.[3]; Dlouhy, Stephen R.[3]; Robertson, Kent [4]; Haut, Paul R.[4]; Cornetta, Kenneth[3]; Abonour, Rafat [1].

1. Medicine, Division of Hematology/Oncology, Hematology Malignancy Program and Stem Cell Transplantation, Indiana University School of Medicine, Indianapolis, IN, USA. 2. Medicine, Division of Biostatistics, Indiana University School of Medicine, Indianapolis, IN, USA. 3. Department of Medical and Molecular Genetics; Department of Pathology and Laboratory Medicine; and Indiana University School of Medicine, Indianapolis, IN, USA. 4. Department of Pediatrics, Division of Pediatric Hematology/Oncology, Indiana University School of Medicine, Indianapolis, IN, USA.

Forty-nine consecutive patients with hematological malignancies (median age, 55 years) received peripheral blood hematopoietic progenitor cells from a matched related or unrelated donor following immunosuppressive dosages of cyclophosphamide and fludarabine. GVHD prophylaxis included cyclosporine +/-mycophenolate mofetil; patients were followed until death or at least 7 months (range, 25-1675 days, median 413 days; survivor median follow-up, 37 months). All patients engrafted without growth factor support and with similar chimerism patterns after related (n=26) or unrelated (n=23) transplantation. No subjects experienced veno-occlusive liver disease, mucositis or post-transplant lymphoproliferative disease and 100-day transplant-related mortality was 12.2%. Grade 3 or 4 acute GVHD occurred in 28.6% a median of 34 days post-infusion and was associated with the development of extensive chronic GVHD and poor overall survival. Chronic extensive GVHD, associated with increased donor and recipient age, occurred in 57.2%. One-year and two-year overall survivals were estimated to be 63% and 44.4% by Kaplan-Meier survival analysis. Twenty-four (49%) of patients are alive at a median of 413 days post-transplant; 23 (95.8%) in complete remission. Rates of aGVHD, cGVHD, and disease-free or overall survival were not statistically different between recipients of MRD and MUD grafts. Survivor mean ECOG performance status is 0.41; eleven of fourteen survivors with chronic GVHD are requiring decreased immunosuppressive therapy or have experienced resolution. These findings provide evidence for long-term disease-free survival and tendency for resolution of cGVHD after truly nonablative transplantation for hematological malignancies in older patients.

Early Expansion of Lymphoid Cells Precedes Myeloid Engraftment Following Hematopoietic Cell Transplantation Using Truly Nonmyeloablative Cyclophosphamide/Fludarabine Conditioning

Ananda V. Pandurangadu, M.S., Jay Whan, Menggang Yu, Ph.D. Robert P. Nelson, Jr. M.D.,

Corresponding author:

Robert P. Nelson, Jr. M.D.
Associate Professor of Medicine
Division of Hematology/Oncology
Hematological Malignancy Program/Immunology and
Stem Cell Transplantation Service
Indiana University School of Medicine

Abstract: Nonmyeloablative allogeneic transplantation for hematological malignancies is associated with high engraftment rates, disease responses and reduced toxicities compared to myeloablative transplants. Little is known about the mechanism of engraftment after such transplants.

Purpose/Background: Characterize white blood cell and differential engraftment patterns after allogeneic transplantation using nonmyeloablative cyclophosphamide/ fludarabine conditioning.

Methods: Routine complete blood counts and differentials were obtained from 49 consecutive patients with hematological malignancies who received a matched donor graft following uniform nonmyeloablative conditioning. Results of daily CBCs from 7 days prior to transplant (day –7) through twenty days post-transplant (day +20) were collected. Polymorphonuclear leukocytes, monocytes and lymphocyte percentages and absolute counts were analyzed.

Results: To be presented.

Mobilization of Peripheral Blood CD34 stem cells in a Heavily Pre-treated Pediatric Medulloblastoma Patient using AMD3100 and G-CSF.

Kent Robertson[1] , Kamnesh Pradhan[1] , Scott Goebel[1] , Jamie Renbarger[1] , Rafat Abonour[1] , Gary Calandra[2] , Ron McFarland[2] , and Paul Haut[1] .
[1] Indiana University School of Medicine, Indianapolis, Indiana, United States and
[2] AnorMED Inc, Langley, British Columbia, Canada.

Autologous peripheral blood stem cell transplantation has been beneficial in the setting of recurrent medulloblastoma, however many of these patients are heavily pre-treated making conventional mobilization of peripheral blood stem cells with G-CSF alone difficult. The recent development of AMD3100 as an inhibitor of the binding of SDF-1/CXCL12 to its receptor CXCR4 in the marrow stem cell compartment, has resulted in significant enhancement in the mobilization of peripheral blood stem cells. This has met with considerable success in adults with phase III trials under way, however there is little pediatric experience with the use of AMD3100. We report here the use of AMD3100 to mobilize peripheral blood CD34 cells from a heavily pre-treated 11 yo girl with recurrent medulloblastoma. DM was diagnosed with stage IV medulloblastoma in Feb 2004 and was treated with surgical resection, radiation, and maintenance chemotherapy including cisplatin, CCNU, and vincristine. Prior to her 5th cycle of chemotherapy she experienced a (biopsy proven) relapse. She went on to receive salvage chemotherapy including carboplatin and cytoxan with stabilization of disease, but without shrinkage in her tumor. Attempts to collect peripheral blood stem cells upon recovery from chemotherapy using G-CSF at a dose of 24mcg/kg/day were unsuccessful with only 1×10^5 CD-34 positive stem cells per kg collected over 3 days. In order to attempt to collect enough stem cells for an autologous stem cell transplant, we obtained consent from the AnorMED corporation and our institutional review board for a PBSC collection using 4 days G-CSF at 10 mcg/kg sc followed by 240 mcg/kg AMD3100 sc and subsequent apheresis 10 hours later. We collected PBSC's for three days and repeated this cycle a second time 30 days later which allowed us to collect 1.3×10^6 CD34 positive stem cells per kg, more than an order of magnitude greater than with G-CSF alone. There were no untoward toxicities including no GI upset, paresthesias ,or injection site reactions which have been previously reported with adult patients. Pharmacokinetics were performed and will be presented, but were similar to that reported in adults.

Quality Control of Human Tissues-Experience from the Indiana University -Lilly Research Lab Tissue Bank

Sandusky, GE, Powell, EL, Esterman, ME, Hanson, JC, Lahn, MMF, and Williams, SD
Lilly Research Labs, Indiana University School of Medicine Cancer Center, Indianapolis, Indiana 46285

Successful identification and exploration of novel molecular therapeutic targets in clinical and basic research is strongly dependent on the availability of good quality human tissue samples. Collection, handling, and storage conditions are important variables that effect affect the quality of human tissue samples. In genomic and proteomic research, banked human tissue has been managed with the Clinical Annotated Tissue Database (CATD) in order to study genes and proteins which may be up or down regulated in certain cancers when compared to expression in normal human tissues. This abstract is a compilation of the literature involved in the collection, processing, and storage of tissue as well as basic quality control generated from the tissue collected using good clinical practices. The current applicable rules, HIPAA regulations, and guidelines for handling human tissues are briefly discussed.
In this study in the area of genomic and proteomic research, we determined that the high quality of the tissue sample is imperative for both genomic and proteomic molecular research. Approximately 1300 tissue samples were evaluated and the data is presented below. Each piece of tissue (one patient often has multiple 250mg samples) was QC'd by H&E evaluation for tumor content. One sample from each patient was evaluated for RNA integrity. Approximately 74% of the samples contained more than 50% tumor on H&E QC. Detailed patient clinical annotation and patient disease follow-up was stored and retrieved through the web based CATD system. Quality control comprising detailed retrievable data sets that consist of clinical annotation, treatment outcome, and tissue integrity optimizes the validation of novel therapeutic targets in human cancer research.

An Abundantly Expressed Splice Variant of Carboxylesterase-2 Lacks Irinotecan Hydrolase Activity

Marissa A. Schiel, Scheri-lyn Green, Wilhelmina I. Davis, and Sonal P. Sanghani
Department of Biochemistry and Molecular Biology, Indiana University Purdue University at Indianapolis, Indiana 46202

Irinotecan is a carbamate prodrug that is activated *in vivo* by carboxylesterase-2 (CES2) to SN-38, a potent topoisomerase I inhibitor. Significant inter-individual variation has been reported in plasma SN-38 levels after administration of irinotecan in patients. Several studies have reported single nucleotide polymorphisms (SNPs) for CES2 gene. However, no significant relation has been identified between the CES2 SNPs and CES2 RNA expression or irinotecan hydrolase activity. The expressed sequence tag (EST) database indicates that CES2 undergoes several splicing events. One such splice variant, CES2 $^{458-473}$, has a 16 amino acid deletion immediately following the active site histidine. In this study, we report the expression and activity of a CES2 $^{458-473}$ splice variant. We have cloned and expressed the CES2 $^{458-473}$ protein in Sf9 insect cells. The recombinant protein was purified by the same method used for wild-type CES2 protein. Recombinant CES2 $^{458-473}$ protein has been characterized by several methods to ensure its proper folding. Activity assays indicated that the CES2 $^{458-473}$ protein lacks carboxylesterase activity for 4-methyl-umbelliferyl acetate. In order to evaluate its irinotecan hydrolase activity we incubated 0 to 50 g of CES2 $^{458-473}$ variant protein with 55 M irinotecan. SN-38 production was monitored by HPLC. We found that even after 2h incubation of 50 g of CES2 $^{458-473}$ variant with irinotecan we could not detect a peak for SN-38. In a parallel experiment we found that 30 g of wild type CES2 produced about 2 M of SN-38 after 30 min incubation. In addition to enzymatic activity, we were interested in expression levels of the CES2 $^{458-473}$ variant. We probed multi-tissue northern blot with CES2 $^{458-473}$ variant specific oligonucleotide to confirm its expression in normal liver tissue. Using variant specific primers the mRNA levels of CES2 and CES2 $^{458-473}$ variant from 24 colon tumor samples were analyzed by real-time PCR. The results from the real-time PCR indicated that CES2 $^{458-473}$ mRNA accounts for 6.7 to 25.7 % of CES2 in colon tumors. In conclusion, we have found that the CES2 $^{458-473}$ protein is an inactive splice variant of CES2 and that its message accounts for a significant portion of CES2 message in colon tumors. To date CES2 SNP data has not provided any explanation for the high inter-individual variability in response to irinotecan treatment. Therefore, we propose that the expression levels and activity of the CES2 splice variants may be responsible for the inter-individual variability and key factor in determining patient response to irinotecan therapy.

Parthenolide analogue LC-1 induces apoptosis in p53 mutant bladder cancer cell lines by modulating apoptotic genes and enhanced p73 and p21 activation.

Rajasubramaniam Shanmugam[1,2] , Sundar Neelakantan[3], Vetrichelvan Jayaprakasan[1,2], Peter Crooks[3], Harikrishna Nakshatri[2,4,5], Christopher Sweeney[1,2].
[1] Department of Hematology/Oncology, Indiana University School of Medicine, Indianapolis, IN [2] Walther Cancer Institute, Indianapolis, IN, [3] University of Kentucky, Kentucky, [4] Department of Surgery and [5] Department of Biochemistry and Molecular Biology, Indiana University, Indianapolis IN.

Bladder cancer is the fourth and eighth most common cancer in men and women in USA respectively. Platinum based therapy is the standard therapy for metastatic disease despite this most patients die of platinum resistance. The sesquiterpene lactone, parthenolide has been shown to possess anticancer activity and decrease drug resistance. However its bioavailability is low due to its solubility. We assessed the effect of water soluble analogue LC-1 on bladder cancer cells UMUC-3, HT-1376 and HT-1197. Electrophoretic mobility shift assay revealed decrease in Nuclear factor kappa B-DNA binding in a dose dependent manner. LC-1 inhibited proliferation of UMUC-3, HT-1376 and HT-1197 with IC_{50} between 5 and 10μM. Micro-array analysis revealed increased expression of tumor suppressor p73, cell cycle inhibitor p21, death domain TRADD and caspase 3, 8 and 9. Antiapoptotic genes under NFκB control TRAF1 and TRAF 2 were also inhibited. Western blotting of LC-1 treated cells showed dose and time dependent JNK and c-Jun activation in addition to increased caspase activation. p73 mRNA levels increased significantly in cells treated with LC-1. Further, p53 targets – p53 upregulated modulator of apoptosis (PUMA) and p21 were induced in response to LC-1 suggesting p73 replaces p53 in p53 deficient cancer cells. Thus the results suggest that LC-1 modulates proapoptotic genes and tumor suppressor activity providing a strong rationale for further studies

Skin Cancer after Nonmyeloablative Hematopoietic Cell Transplantation

Shmalo, James A.[2]; Cavalier, MaryEllen[3]; Yu, Menggang[4]; Billings, Steven D.[5]; Nelson, Robert P.[1].

1. Medicine, Division of Hematology/Oncology, Hematology Malignancy Program and Stem Cell Transplantation, Indiana University School of Medicine, Indianapolis, IN, USA. 2. Pediatrics, Indiana University School of Medcine, Indianapolis, IN, USA. 3. Department of Pediatrics, Division of Pediatric Hematology/Oncology, Indiana University School of Medcine, Indianapolis, IN, USA. 4. Medicine, Division of Biostatistics, Indiana University School of Medicine, Indianapolis, IN, USA. 5. Pathology, Laboratory Medicine and Dermatology, Indiana University School of Medicine, Indianapolis, IN, USA.

Nonmyeloablative hematopoietic cell transplantation (NMAT) utilizes transient, intensive immunosuppression without marrow ablation that permits allogeneic engraftment. Forty-nine consecutive patients with hematological malignancies 33 (67.3%) males and 16 (32.7%) females underwent NMAT from May 2000 through October 2004, utilizing uniform cyclophophamide/fludarabine conditioning with cyclosporine +/- mycophenolate mofetil graft-versus-host (GVHD) prophylaxis. Median age for all subjects was 55 years (range, 17 to 66 years). All related (n=26, 53.1%) or unrelated (n=23, 46.9%) donors were fully matched at HLA-A, HLA-B, and HLA-DR loci. Patients were followed until death or at least 7 months (range, 25-1675 days; median, 413 days).

Six patients developed 18 skin cancer lesions from 2 to 26 months following NMAT. Cancer types include squamous cell carcinoma (n = 14), basal cell carcinoma (n = 2) and malignant melanoma (n = 2). Pre-transplant treatment intensity was not different between those who developed skin cancer and those who did not. One of the patients with and none of the patients without post-transplant skin cancer had a history of skin cancer prior to transplant. All patients with skin cancer had chronic GVHD that was extensive in all but 1, and 5 (80%) had skin involvement as part of their chronic GVHD diagnosis. There was a skin cancer predilection for patients with diagnoses of acute myelogenous leukemia and myelodysplasia ($p=0.031$). All eighteen lesions were excised without dissemination.

The finding of skin cancer after NMAT in 6 of 49 patients suggests that intensive, relatively short-term immunosuppression does not eliminate post-transplant skin cancer risk and underscores the need for vigilant skin cancer surveillance and recommending regular use of sun block in transplant recipients.

Examination of an IL-12 single-chain peptide under control of a prostate specific enhancer

Catherine Steding

Interleukin-12 (IL-12) has been shown to have potent anti-tumor activity against many different malignancies, including prostate cancer. Previous studies utilizing replication-deficient adenovirus vectors to deliver IL-12 under control of the universal cytomegalovirus (CMV) promoter have shown success as a therapy; however, this system has limited clinical feasibility due to toxic side-effects. Furthermore, the deficiency in viral replication of previously tested adenoviral vectors has resulted in limited therapeutic efficacy due to limited viral gene transduction. For these reasons, a prostate-specific enhancer within a replication competent adenovirus vector has been developed and tested. The prostate-specific PSES enhancer has demonstrated tissue specificity for replication of the adenovirus vector as well as tissue-specific production of various genes. Examination of IL-12 single-chain peptide production and function from adenoviral production plasmids containing PSES prostate-specific enhancer driven expression of the gene was performed to determine its potential use as a tissue specific treatment for prostate cancer. Experimental findings indicate that the PSES enhancer-mediated expression of an adenovirus-based system produces a functional single-chain IL-12 peptide after transfection into a prostate cancer cell line.

Neurofibromin Plays a Critical role in Modulating Osteoblast Differentiation of Mesenchymal Stem Cells

Xiaohua Wu, Selina A. Estwick, and Fengchun Yang
Herman B. Wells Center for Pediatric Research, Department of Pediatrics
Indiana University School of Medicine, Indianapolis, IN 46202

Mutations in the *NF1* tumor suppressor gene cause neurofibromatosis type 1, a pandemic autosomal dominant genetic disorder with an incidence of 1:3500. Individuals with NF1 have a variety of malignant and non-malignant manifestations, including skeletal abnormalities, such as osteoporosis, scoliosis, and short statures. However, the mechanism(s) underlying the osseous manifestations in NF1 are poorly understood. In the present study, utilizing *Nf1* haploinsufficient (+/-) mice, we demonstrate that *Nf1+/-* mesenchymal stem cells (MSC) have increased cell growth, proliferation, and colony forming unit-fibroblast (CFU-F) activity compared to wildtype (WT) MSC. *Nf1+/-* MSC also have fewer senescent cells and have a significantly higher telomerase activity compared to WT MSC. Furthermore, *Nf1+/-* MSC showed impaired osteoblast differentiation as determined by alkaline phosphatase (ALP) staining and adipogenic differentiation evidenced by Oil Red O staining. These results further confirmed by single CFU-F replating assays. However, no difference was found in chondrogenic differentiation beween WT and Nf1+/- MSC. The impaired osteoblast differentiation and adipogenic differentiation in *Nf1+/-* MSC were consistent with the reduced expression of osteoblast markers at the mRNA level, including osteocalcin and osteonectin, and adipocyte markers, including PPARγ1, PPARγ2, and lipoprotein lipase. Most importantly, re-expression of the full-length *NF1* GTPase activating related domain (*NF1* GRD) was sufficient to reduce the Ras-GTP activity to the control level and restore the impaired osteoblast differentiation in *Nf1+/-* MSC. Taken together, our results suggest that the neurofibromin is involved in MSC biological functions and GRD domain of neurofibromin plays a critical role in modulating MSC differentiation into osteoblasts, which may contribute at least in part to the osseous abnormalities seen in individuals with NF1.

HOXB13 is Down-regulated in Colorectal Cancer to Confer TCF4-mediated Transactivation

Hongji Zhang[1], Hongmiao Sheng[2], Patrick J. Loehrer[3], Thomas A. Gardner[1,4], Meei-Huey Jeng[3,4], Chinghai Kao[1,4], and Chaeyong Jung[1]

[1]Departments of Urology, [2]Surgery, [3]Medicine, and [4]Microbiology and Immunology, Indiana University, Indianapolis, IN 46202, USA

Mutations in the Wnt signaling cascade are believed to cause aberrant proliferation of colorectal cells by T cell factor-4 (TCF4) and its downstream growth-modulating factors. HOXB13 is exclusively expressed in prostate and colorectum. In prostate cancers, HOXB13 negatively regulates β-catenin/TCF4-mediated transactivation and subsequently inhibits cell growth. To study the role of HOXB13 in colorectal tumorigenesis, we evaluated the expression of HOXB13 in 53 colorectal tumors originated from the distal left colon to rectum with their matching normal tissues using quantitative RT-PCR analysis. Expression of HOXB13 is either lost or diminished in 26 out of 42 valid tumors (62%), while expression of TCF4 RNA is not correlated with HOXB13 expression. TCF4 promoter analysis showed that HOXB13 does not regulate TCF4 at the transcriptional level. However, HOXB13 down-regulated the expression of TCF4 and its target gene, c-myc, at the protein level and consequently inhibited β-catenin/TCF-mediated signaling. Functionally, forced expression of HOXB13 drove colorectal cancer cells into growth suppression. This is the first description of down-regulation of HOXB13 in colorectal cancer and its mechanism of action is mediated through the regulation of TCF-4 protein stability. Our results suggest that loss of HOXB13 may be an important event for colorectal cell transformation, considering that over 90% of colorectal tumors retain mutations in the APC/β-catenin pathway.

Submitted by: Chaeyong Jung chjung@iupui.edu

BRMS1: Metastasis Suppressor Gene Methylation as Target for Anticancer Therapy in Breast and Ovarian Cancer

Shu Zhang, Curtis Balch, Meiyun Fan, and Kenneth P. Nephew

Indiana University, Medical Science Program, School of Medicine, Bloomington, Indiana 47405

The BRMS1 (breast cancer metastasis suppressor 1) gene has been found to suppress metastasis in animal models without inhibiting primary tumor growth. Our prior work indicated that reduced *BRMS1* expression is associated with aggressive ovarian carcinomas, nodal metastases and poor patient survival. Because low *BRMS1* RNA expression correlate with poor clinical course, we have focused on the regulation of *BRMS1* at the transcriptional level. One notable mechanism of transcriptional regulation and gene silencing is that hypermethylation of CpG islands within the first exon and promoter regions of tumor and metastasis suppressor. Based on the observation that high-density CpG island is present in *BRMS1* promoter, we hypothesize that DNA methylation regulates *BRMS1* expression on breast and ovarian cancer. We further hypothesized that treatment with 5-Aza-2'-deoxycytidine (5-Aza-CdR), a DNA methylation inhibitor, will increase *BRMS1* expression in breast and ovarian carcinoma cell lines. Metastasis suppressor gene reactivation may inhibit metastatic colonization and further invasion, resulting in clinical benefit. *BRMS1* mRNA levels were examined in a panel of breast and epithelial ovarian cancer cell lines and normal ovarian surface epithelial cells (NOSE) by real time quantitative PCR (qPCR) or as part of a microarray analysis performed on these cell lines. Hypermethylation of the *BRMS1* CpG island in these cell lines was determined by methylation specific PCR (MSP). Increased *BRMS1* expression and decreased motility of metastatic cell lines HO-8910PM ovarian cancer and MDA-MB-231 breast cancer were observed following treatment with 5-Aza-CdR. Our qPCR and microarray analyses confirmed the *BRMS1* mRNA expression in HO-8910PM and ICI-R antiestrogen resistant cell (derived from MCF-7 breast cancer) was significantly lower than those in NOSE and MCF-7 respectively ($P < 0.05$). The down regulation of BRMS1 in ICI-R breast cancer is accompanied with the up regulation of some pro-metastasis gen including MMPs, suggesting ICI-R get capability to metastases. Down regulation of *BRMS1* expression was also observed in MDA-MB-231 breast cancer cell lines. In matrigel invasion assay, increased *BRMS1* expression and decreased motility of metastatic cell lines HO-8910PM ovarian cancer and MDA-MB-231 breast cancer were observed. In the HO-8910PM ovarian carcinoma animal model system, over-expression of *BRMS1* resulted in a 80% inhibition of metastasis *in vivo.* Our preliminary results suggest that DNA methylation may contribute to the regulation of *BRMS1* expression levels. Collectively, the results indicate that *BRMS1* could provide a new and important target for therapeutic intervention of breast and ovarian cancer, and DNA methylation inhibitor may hold promise in increasing metastasis-suppressor gene expression.

Plasma lysophosphatidylcholine levels: potential biomarkers for colorectal cancer

Zhenwen Zhao[1,2,§], Yijin Xiao, PhD[2,3,§], Paul Elson, ScD[4], Haiyan Tan, MD[2], Sarah J. Plummer, BS[2], Michael Berk, MS[2,5], Ian C. Lavery, MD[6], Jean Paul Achkar, MD[7], Graham Casey, PhD[2,4,6,8], and Yan Xu, PhD[1,2,8*]
[1]Division of Gynecologic Oncology, Department of Obstetrics and Gynecology, Indiana University Cancer Center, Indiana University School of Medicine, 975 W. Walnut St. IB355A, Indianapolis, Indiana;[2]Department of Cancer Biology, Lerner Research Institute, The Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, OH 44195; [3]Frontage Laboratories, Inc., 105 Great Valley Parkway, Malvern, PA 19355; [4]Taussig Cancer Center, The Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, OH 44195; [5]Current address: The General Clinical Research Center Core Laboratory; [6]Department of Colorectal Surgery; [7]Department of Gastroenterology; [8]Department of Gynecology and Obstetrics, The Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, OH 44195.
[§] These two authors contributed equally to this work.

Abstract

Colorectal cancer (CRC) is the fourth most commonly diagnosed cancer and the second leading cause of cancer-related deaths in the United States. Accurate and convenient early detection of CRC is critical for reducing mortality. Plasma lysophospholipid levels have been shown to be promising diagnostic markers for some cancers. In this work, Plasma samples from 138 CRC cases, 13 patients with adenomas, and 125 unaffected controls were analyzed for plasma lysophospholipid levels using liquid chromatography mass spectrometry (LC-MS). All assays were performed blinded to disease status. Comparisons between subject groups were analyzed using nonparametric methods. Logistic regression models were used to identify markers that could discriminate between CRC/adenoma cases and controls. Receiver operating characteristic (ROC) curves were used to summarize the overall accuracy of the models. Validation of the final model was conducted using a bootstrap re-sampling approach. It was concluded that plasma levels of several lysophosphatidylcholine (LPC) species, especially 18:1- and 18:2-LPC, were significantly decreased in CRC and adenoma cases, compared to unaffected controls (P<.001). Using several LPC species as markers, we obtained 95% specificity and 84% sensitivity in the classification of CRC and adenomas versus unaffected controls. In particular, 115/138 (83%) CRC cases and 12/13 (92%) of adenoma cases were correctly identified as “diseased” cases and 119/125 cases were correctly identified as healthy control cases. Considering the inconvenience of colonoscopy test due to its invasive nature and the low sensitivity (about 20%) of fecal occult blood test (FOBT), 18:1- and 18:2-LPC plasma levels alone or in combination with total saturated LPC levels may behave more potential for the detection of CRC and adenomas.

*Corresponding author: Division of Gynecologic Oncology, Department of Obstetrics and Gynecology, Indiana University Cancer Center, Indiana University School of Medicine, 975 W. Walnut St. IB355A, Indianapolis, Indiana xu2@iupui.edu

POPULATION

A description of Local IRB variation and its Impact on a Multi-Site Behavioral Oncology Trial

Joan E. Haase, PhD, RN, Holmquist Professor in Pediatric Oncology Nursing,
Indiana University School of Nursing

PURPOSE: The increasing number of multi-site studies and the differences in the policies and procedures across sites requires a complex and costly process to obtain IRB approvals. The numerous layers of approval and differences in timelines and different requirements for types of approval and informed consent and adverse event documentation often contribute to delay in patient accrual, proposed study timelines, and increased start-up expenses. There is scant literature documenting the complexity and processes to obtain IRB approval for a multi-site study. The purpose of this project is to describe the variability in local Institutional Review Board (IRB) and other regulatory oversight processes required and their impact on the start-up of an NIH-funded multi-site behavioral oncology clinical trial.

METHODS: Data on IRB variabition were obtained from 3 sources in a 6-site NIH-funded study of adolescent/young adults undergoing stem cell transplant. : 1) minutes of bi-weekly site principal investigator (PI) meetings; 2) a survey completed by site PI's; and, 3) a systematic examination of requirements at each site. A matrix comparison of documentation, layers of approval (i.e. cancer center, nursing committees, etc.), and policies and procedures across institutions was done.

RESULTS: Findings indicate that most approving bodies require similar kinds of information in the initial review. However, there was large variability in: 1) the format and level of detail required; 2)informed consent content and forms (one site required 5 different assent/consent forms); and the amount and nature of information exchange allowed between site PI's and compliance staff and reviewers. These elements, coupled with the multiple review levels and differing timelines at each layer within each participating institution create a complex interplay for start-up and promote inconsistency within the study.

CONCLUSION: The variability in IRB requirements across sites is large and has implications for study integrity, timeliness, and costs. Providing a standardized form for all approval layers within an institution and amongst local IRB's would greatly impact the time and cost burden of start-up. These elements are contributing factors to the efficiency and overall success of a multi-site study.

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UNCERTAINTY IN ADOLESCENTS WITH CANCER: A DESCRIPTIVE STUDY.

Carol L. Decker, PhD, MSW, Cynthia J. Bell, BSN, RN & Joan E. Haase, PhD, RN, Indiana University, Indianapolis, IN

Purpose: The purpose of this study was to examine differences in levels of perceived uncertainty for adolescents with cancer (AWC).
Methods: Descriptive study of 198 adolescents with cancer. Data were obtained from two Adolescent Resilience Model (ARM) studies. ARM 1 (1992) consisted of AWC survivors seen at multiple cancer center sites (diagnosed from 11-21 years old). ARM 2 (2003) consisted of newly diagnosed AWC obtained at multiple cancer sites (ages 11-19). The sample consisted of 109 males and 89 females, grouped into three time-since-diagnosis groups: Less than 1 year (N=81); 1-3 years (N=39); and 4 or more years (N=78). Uncertainty was measured using Mishel's Uncertainty in Illness Scale (MUIS) α=.91 as part of a booklet of self-administered instruments. MUIS is a Likert-type scale with 33.items. Responses to statements such as "I can predict how long my illness will last" range from 1=strongly agree to 5=strongly disagree. Higher scores indicate higher uncertainty, after reversal of negatively worded items
Results: Based on Chi Square analysis, no significant age or gender differences were found for the three time-since-diagnosis groups. Using ANOVA, no significant differences in levels of uncertainty for gender, age, or time-since-diagnosis were found. Examination of individual scale items revealed significant time-since-diagnosis group differences for eight items. Compared to the other two groups, newly diagnosed AWC had significantly higher uncertainty on items about future pain, unpredictable symptoms, understanding different staff responsibilities, and concerns about caring for themselves. Newly diagnosed AWC had significantly lower uncertainty on items related to the probability of successful treatment, future diagnoses, knowing what is wrong, and having unanswered questions (latter 3 items significant only compared to those diagnosed 4+ years). AWC 1-3 years from diagnosis had significantly lower uncertainty on concerns for future diagnoses compared to the other two groups. The only gender differences for individual items showed males having higher uncertainty for knowing what was wrong with them. No age differences were detected for individual items.
Conclusions: Uncertainty has been described as a major source of stress for patients facing a life-threatening illness Regardless of time since diagnosis; AWC had similar, moderate levels of overall uncertainty. This was true for AWC even years after the end of treatment. The time-since-diagnosis group differences for individual items provides additional information for healthcare providers. AWC need different types of reassurance and information depending on the time since diagnosis. Newly diagnosed AWC need reassurance. Their information needs pertain to immediate issues, i.e. staff responsibilities, symptoms, regaining self-care and potential pain. AWC 1-3 years from diagnosis remain closely connected to the medical providers, as reflected in their understanding of staff responsibilities and lower concerns about a new diagnosis. AWC 4+ years from diagnosis have the highest uncertainty related to diagnosis and unanswered questions, indicating their need for continued opportunities to ask questions as well as to obtain information about potential late effects, recurrence, or a secondary malignancy.

Assessing Health Beliefs About Sigmoidoscopy

Michelle Mabis, Research Assistant, Susan Rawl, PhD, RN, Associate Professor, Connie Krier, BS, Project Manager, Patrick Monahan, PhD, Biostatician, Honghong Zhou, PhD, Biostatician, and Yan Ding, MS, Biostatician. Indiana University – Purdue University at Indianapolis (IUPUI), 1111 Middle Drive, Indianapolis, IN 46202
Biographical sketch of first author: Michelle Mabis is a seventh semester undergraduate student in the Indiana University School of Nursing. She was instrumental in recruiting subjects and collecting data by telephone interview for the NIH-funded behavioral oncology study *Telehealth Intervention for Colorectal Cancer Screening*.

Colorectal cancer (CRC) is the second leading cause of cancer-related deaths with an estimated 148,610 new cases and 55,170 deaths in 2006. Colorectal cancer is highly preventable with appropriate screening and removal of adenomatous polyps. Mortality can be reduced by 60% with sigmoidoscopy for cancers within reach of the scope. Because awareness of the sigmoidoscopy as a CRC screening test is low, challenges arise in evaluating beliefs about this test. The purpose of this study was to examine the influence of experience with sigmoidoscopy on assessment of perceived benefits and barriers to this test. Baseline data were collected via telephone interview from 139 relatives of patients who had been diagnosed with colon polyps. The Health Belief Model guided this study with relevant constructs (perceived risk, benefits, barriers, self-efficacy) assessed separately for fecal occult blood test, sigmoidoscopy, and colonoscopy. Results indicated that, while awareness of fecal occult blood tests and colonoscopy were near universal, only 58% of participants had ever heard of a sigmoidoscopy and less than 11% had ever had one. Using independent samples t-tests, we examined differences between participants who had prior experience with sigmoidoscopy (n=15) and those who were naïve to sigmoidoscopy (n=124) in health beliefs. Participants who had no prior experience with sigmoidoscopy had higher levels of agreement with "*I don't know what will happen during a sigmoidoscopy*" and "*I am afraid to have a sigmoidoscopy because I might find something wrong*". Those who were experienced with sigmoidoscopy had higher levels of agreement with "*sigmoidoscopy is painful*" and "*sigmoidoscopy will help me not worry as much about CRC"*. We concluded that perceived benefits of and barriers to sigmoidoscopy may be influenced by experience with the test. Reliable and valid assessment of perceived benefits and barriers to sigmoidoscopy among CRC screening-naïve persons remains a challenge.

A Litany of Symptoms from the Mind, Body, and Soul: Breast Cancer Survivorship in African American Women

Kathleen M. Russell, DNS, RN; Joan E. Haase, PhD, RN; Wendy Kooken, MSN, RN; Yueh-Feng Yvonne Lu, PhD, RN; R Brian Giesler, PhD; Kim Wagler Ziner, MSN, RN

A complex picture of factors affects adaptation following diagnosis and treatment of breast cancer for African American women survivors. African American women often describe health as an integration of the mind, body, and spirit. Yet, little is known about these views in the context of cancer survivorship. The purpose of this study was to gain a clearer understanding of these aspects of breast cancer survivorship for African American women and to identify how these factors affected their perceived quality of life. We conducted 3 focus groups with 21 African American women across socioeconomic strata who were breast cancer survivors from 1 to 10 years post initial treatment. Using a phenomenological analysis combined with group-as-a-whole theory, we found even though experiences differed among the women, the experiences emerged around the following clusters: (1) a litany of physical symptoms, (2) mixed emotional responses, including vulnerability, shame, fear and being pulled in different directions, and (3) a very strong connection to a higher being with conflicting feelings. Findings point to closely examining our approaches to care when addressing needs of African American women breast cancer survivors and to integrating and tailoring interventions in their physical, psychosocial and spiritual domains. Further research also is needed to assure that our current quality of life measures are inclusive of African American women.

Goal
Increase understanding about varying responses from the physical, emotional, and spiritual domains to quality of life in African American women breast cancer survivors.

Objectives

1. Describe the physiological, psychosocial, and spiritual responses to breast cancer survivorship in African American women.
2. Discuss similarities and differences in responses to breast cancer survivorship.
3. Consider ways to promote wellness in breast cancer survivorship in African American women.

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Telehealth Nursing Intervention to Promote Colon Cancer Screening: Participant Recall and Satisfaction

Karen Schmidt, RN, MSN, Research Assistant[1], Susan Rawl, PhD, RN, Assistant Professor[1], Victoria Champion, DNS, RN, FAAN, Associate Dean for Research[1], Connie Krier, BS, Clinical Research Coordinator[1], Michelle Symms, RN, BSN, Research Assistant[1], Michael Burns, RN, BSN, Research Assistant[1], Eileen Kramer, BA, Research Analyst[2], Mariano Erpe, BS, Research Analyst[2], and Douglas Rex, MD, Professor[2]. (1) Indiana University Purdue University at Indianapolis, 1111 Middle Drive, Indianapolis, IN 46202, fax: 317-278-2021, (2) Indiana University, 1111 Middle Drive, Indianapolis, IN 46202
Biographical sketch of first author: Karen Schmidt, RN, MSN is providing telehealth nursing interventions and recruiting for several studies focused on increasing colorectal cancer screening.

In 2004, an estimated 146, 940 people will be diagnosed with colorectal cancer and 56,730 will die from the disease. The majority of these deaths could be prevented since colorectal cancer (CRC), when discovered early, is highly treatable. Population screening for CRC has the potential to reduce the mortality rate by 50%. Screening that leads to removal of adenomatous polyps, the precursors to CRC, has been found to decrease incidence of this disease by 75% to 90%. Unfortunately the prevalence of CRC screening remains low. Nationally, only 50% of adults aged 50 or older reported having had any screening test in the recommended intervals. Tailored telephone counseling, one specific type of telehealth intervention, has demonstrated considerable promise as an intervention that motivates people to change health behaviors. We have developed a tailored telephone counseling intervention designed to promote CRC screening among relatives of persons diagnosed with colon polyps. The tailored telephone counseling intervention, delivered by trained nurses, contains individually tailored messages for each participant based on baseline assessment of their individual demographics, CRC risk factors, Health Belief Model constructs of perceived CRC risk, perceived benefits, barriers, self-efficacy, and stages of change for fecal occult blood testing, sigmoidoscopy and colonoscopy. A larger, ongoing randomized trial will compare the efficacy of the tailored telephone intervention with a mailed generic print intervention. In this study, we will examine recall and satisfaction with the tailored telephone counseling versus a generic mailed brochure. Data were collected at three months post-intervention via structured telephone interviews. Group differences will be analyzed using t-tests for continuous variables and chi-square analyses for categorical data. Results from this study will advance nursing knowledge of telehealth interventions as a health promotion and disease prevention tool.