

BASIC SCIENCE

Indoleamine 2,3-dioxygenase controls conversion of Foxp3⁺ Tregs to TH17-like cells in tumor-draining lymph nodes

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The immunoregulatory enzyme indoleamine 2,3-dioxygenase (IDO) is expressed by a subset of murine plasmacytoid DCs (pDCs) in tumor-draining lymph nodes (TDLNs), where it can potently activate Foxp3⁺ regulatory T cells (Tregs). We now show that IDO functions as a molecular switch in TDLNs, maintaining Tregs in their normal suppressive phenotype when IDO was active, but allowing inflammation-induced conversion of Tregs to a polyfunctional T-helper phenotype similar to proinflammatory T-helper-17 (TH17) cells when IDO was blocked. In vitro, conversion of Tregs to the TH17-like phenotype was driven by antigen-activated effector T cells and required interleukin-6 (IL-6) produced by activated pDCs. IDO regulated this conversion by dominantly suppressing production of IL-6 in pDCs, in a GCN2-kinase dependent fashion. In vivo, using a model of established B16 melanoma, the combination of an IDO-inhibitor drug plus antitumor vaccine caused up-regulation of IL-6 in pDCs and in situ conversion of a majority of Tregs to the TH17 phenotype, with marked enhancement of CD8⁺ T-cell activation and antitumor efficacy. Thus, Tregs in TDLNs can be actively reprogrammed in situ into T-helper cells, without the need for physical depletion, and IDO serves as a key regulator of this critical conversion. Pharmacologic IDO inhibitor 1-methyl-D-tryptophan (1MT) is now in Phase I clinical trials and Phase II trials are planned. The synergy between 1MT and vaccines in the mouse model, with the mechanistic explanation of converting Tregs into TH17 cells, gives a strong molecular rationale for testing 1MT+vaccines in Phase II clinical trials.

What Can Brown Do For You? The Many Roles of the Ubiquitin-Proteasome System (UPS) and Monoubiquitination in Regulating Mammalian Transcription

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Major histocompatibility class II (MHC II) molecules are glycoproteins that present extracellular antigens to CD4⁺ T cells and are essential for initiation of adaptive immune response. MHC II molecules are critical for tumor recognition as multiple highly metastatic tumors down regulate the expression of MHC II to evade activation of the anti-tumor inflammatory response. Because MHC II is regulated solely at the level of transcription, it is important to understand MHC II gene expression in order to aid in the development of novel anti-tumor therapies. MHC II expression requires recruitment of a master regulator, the class II transactivator (CIITA), to the MHC II promoter. We have previously linked CIITA to the UPS by demonstrating that monoubiquitination of CIITA dramatically increases its transactivity whereas polyubiquitination leads to CIITA degradation. The 26S proteasome, the master regulator of protein degradation, has also been shown to have non-proteolytic roles in transcription. We have shown that 19S ATPase subunits of the 26S proteasome regulate MHC II transcription and are necessary for stable promoter binding of CIITA. Here, we identify an ATPase binding domain in the N-terminus of CIITA that is crucial for CIITA stability and MHC II expression. Furthermore, we have identified phosphorylation and ubiquitination sites in CIITA that are critical for CIITA transactivity and MHC II expression. An understanding of the proteolytic and non-proteolytic role of the UPS in MHC II transcription will provide novel strategies for manipulating the expression of MHC II genes and thus aid in the development of novel anti-tumor therapies.

Funding: Research supported by the American Cancer Society, the Georgia Cancer Coalition, and the Molecular Basis of Disease Area of Focus at Georgia State University.

Genome-wide DNA Methylation Maps in Follicular Lymphoma Cells.

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We developed a large-scale bisulfite sequencing approach for analyzing genome-wide DNA methylation patterns by combining bisulfite treatment of genomic DNA with single-molecule-based parallel sequencing. The methylated DNA fragments from lymphoma cells were isolated and enriched using Methylated CpG Islands Recovery Assay (MIRA) which is based on the high affinity of the MBD2b and MBD3L1 proteins complex for methylated DNA. The methylation-enriched genomic DNA was treated with bisulfite and amplified by PCR with primers designed to amplify DNA molecules carrying bisulfite-modified adapter sequences at both ends. The PCR amplicons were sequenced using the Roche-454 GS FLX sequencer. We generated 516K mappable bisulfite sequencing reads (approximately 100Mb data) with an average read length of 143bp (range 20bp to 444bp). Among the 516K bisulfite sequences approximately 436K reads (85%) were uniquely mapped to the human genome. The total number of bases covered on the genome was 18.6 million including 5.4 million cytosines and 739,260 CpGs. We identified 11,972 methylated regions of interests with an average methylation index above 20%. These methylation hot-spots were associated with 4,033 CpG islands (CGIs) that include CGIs associated with several large gene clusters such as HOX and Protocadherin gene clusters. The genome-wide DNA methylation patterns were correlated with transcriptome data from Illumina Beads arrays and ChIP-on-Chip analyses of genome-wide histone modifications such as tri-methyl-H3K27, and tri-methyl-H3K4. These integrated approaches have led to the discovery of novel targets for aberrant DNA methylation in the lymphoma epigenome and provided a comprehensive analysis of the DNA methylation sequence composition and distribution.

To metastasize or not to metastasize – roles for epigenetics in tumor metastasis

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Epigenetic regulation is an essential mechanism by which cells regulate accessibility to chromatin DNA, hence allowing critical processes such as DNA repair or gene transcription to occur. The contribution of dysregulated epigenetic states to specific human cancers remains unknown. In order to achieve metastatic ability, tumor cells alter gene expression to escape from host immunosurveillance. Major histocompatibility class II (MHC-II) molecules are glycoproteins which present tumor derived antigens to CD4⁺ T cells and activate anti-tumor immune responses in order to limit tumor growth. MHC-II molecules are regulated at the level of transcription by a master regulator, the Class II Transactivator, CIITA, whose association with the MHC-II promoter is necessary to initiate MHC-II transcription. Thus, MHC-II are crucial for initiation, regulation and maintaining the anti tumor immune response. To further understand the role played by epigenetics in regulating CIITA and MHC-II expression in metastatic tumor cells, we have investigated histone modifications to CIITA and MHC-II promoters which result in opposing gene expression patterns in three variants of the metastatic breast cancer line MDA-MB435. The MHC-II was down-regulated in highly metastatic variant that shows elevated levels of H3K27 trimethylation indicating closed chromatin structure. Epigenetic changes are now thought to play important roles in the cancer progression. Development of compounds targeting enzymes that regulate histone modifications can be a future of cancer treatment.

EPLIN is a putative invasion suppressor in prostate cancer

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Acquisition of migratory and invasive capabilities by cancer cells is the first step in metastasis, resembling epithelial-to-mesenchymal transition (EMT). It remains elusive whether EMT plays an important role in prostate cancer (PCa) progression. We have established the ARCaP experimental model closely mimicking PCa EMT and clinical bone metastasis. Here, unbiased comparative proteomics analyses were performed in low- and highly-invasive ARCaP cells to identify important proteins involved in PCa EMT. Intriguingly, Epithelial Protein Lost In Neoplasm (EPLIN), a key protein in the stabilization of the cadherin-catenin adhesion complex and actin cytoskeleton, was found to be dramatically reduced upon EMT. EPLIN depletion in PCa cells resulted in significant remodeling of the actin cytoskeleton, transition to a mesenchymal morphology, and enhanced capabilities of *in vitro* migration and invasion. Microarray analyses identified a subset of EPLIN-regulated genes involved in EMT and tumor invasion. Importantly, differential expression of EPLIN was found to be associated with clinical PCa progression and metastasis. Mechanistic studies revealed that prostatic tumor microenvironment, presumably mediated by epidermal growth factor (EGF), induced progressive downregulation of EPLIN, promoted EMT, activated β -catenin signaling, facilitated actin cytoskeleton reorganization, and enhanced migration and invasion. Downregulation of EPLIN was further associated with EMT and clinical progression of human head and neck cancer towards lymph node metastasis. These findings indicate a novel role of EPLIN as an invasion suppressor in certain epithelial cancers.

Hoxc9 is a key mediator for retinoic acid-induced cell cycle arrest and differentiation of human neuroblastoma cells

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Retinoic acid, a derivative of vitamin A, has a key role in vertebrate morphogenesis, cellular differentiation, and tissue homeostasis. In the nervous system, it is involved in the induction of neural differentiation and patterning. It has long been observed that retinoic acid can cause arrest of cell proliferation and differentiation of human neuroblastoma cell lines. As a result, retinoic acid has been in clinical trials as a differentiation therapy for high-risk neuroblastoma, a common childhood malignant tumor of the sympathetic nervous system. The molecular mechanism underlying retinoic acid-induced differentiation of neuroblastoma cells remains poorly understood. Here we report that retinoid acid-induced differentiation of neuroblastoma cells is associated with downregulation of mitotic cyclins. Microarray gene expression profiling identifies the homeobox gene Hoxc9 as one of the genes that are upregulated during the differentiation process. Similar to retinoic acid treatment, ectopic expression of Hoxc9 causes growth arrest, terminal differentiation and senescence of neuroblastoma cells by repressing the expression of mitotic cyclins. Moreover, Hoxc9 knockdown in neuroblastoma cells compromises the ability of retinoic acid to downregulate mitotic cyclins and to induce differentiation. These findings reveal a cellular function of Hoxc9 in the control of mitotic cyclin levels and cell cycle progression, and suggest a molecular mechanism for the action of retinoic acid in neural differentiation.

Axon guidance molecule Slit3 is a novel angiogenic factor

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Slits are large, secreted repulsive axon guidance molecules. Recent genetic studies revealed that the Slit3 is dispensable for neural development, but required for non-neuron-related developmental processes, such as the genesis of organs of diaphragm and kidney. Here we report that Slit3 potently promotes angiogenesis, a process essential for proper organogenesis during embryonic development. We observed that Slit3 is expressed and secreted by both endothelial cells and vascular smooth muscle cells in vasculature, and that the Slit cognate receptors Robo1 and Robo4 are universally expressed by endothelial cells, suggesting that Slit3 may act in paracrine and autocrine manners to regulate endothelial cells. Cellular function studies revealed that Slit3 stimulates endothelial cell proliferation, promotes endothelial cell motility and chemotaxis via interaction with Robo4, and accelerates endothelial cell vascular network formation *in vitro* with a specific activity comparable to vascular endothelial growth factor. Furthermore, Slit3 stimulates neovessel sprouting *ex vivo* and new blood vessel growth *in vivo*, and *Slit3* knockout mice exhibit disrupted vascular development in diaphragm. Taken together, our studies demonstrate that the repulsive axon guidance molecule Slit3 is a novel and potent angiogenic factor, and suggest that Slit3 functions to promote angiogenesis in coordinating organogenesis during embryonic development.

Leptin regulation of VEGF in breast cancer cells.

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We have previously reported that *in vitro* and *in vivo* leptin signaling mediates proliferation of mouse 4T1 and human MCF-7 and MDA-MB231 breast cancer (BC) cells and levels of VEGF and VEGFR2. Specific antagonists of leptin signaling, PEG-LPrAs, were successfully produced and tested in mouse models for BC in our laboratories. Here we present data on how leptin signaling regulates VEGF gene in mouse BC cells (4T1, EMT6 and MMT). BC cells were firstly characterized for expression of VEGFR2 and leptin and estrogen receptor and transiently transfected with VEGF-promoter Luc-reporters [full-length & transcription factor (TF)-binding deletions]. Leptin dose-response, hypoxia and TF and kinase inhibitor effects on reporter activity, signaling pathways, TF and VEGF levels (protein and mRNA) were investigated. Our data suggest that leptin signaling can regulate the transcriptional activity of VEGF gene in breast cancer cells by activating gene transcription at several sites of the VEGF promoter. Leptin-induced PI-3K/AKT1 and NF κ B and HIF-1 α are relevant for the regulation of VEGF in BC. However, leptin activation of NF κ B for VEGF expression has different impact in BC cells. Leptin activation of MAPK/ERK 1/2 and AP1/SP1 was linked to the expression of VEGF. These results delineate the mechanisms for leptin regulation of VEGF and reinforce that the disruption of leptin signaling could impact BC growth by angiogenesis. This further supports the potential use of PEG-LPrA2 for leptin-signaling inhibition for prevention/treatment of BC that could be important for post-menopausal and obese women that show the higher levels of leptin and greatest risk for BC.

Enigma of BRCAness and ER-negative /positive Breast Cancers

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BRCA1 dysfunction is associated with Triple Negative Breast cancers (TNBC). We have identified a consensus SUMO modification site in the amino-terminal region of BRCA1 /1a /1b proteins and mutation in this potential SUMO acceptor site (K 109 to R) impaired their ability to bind and repress ligand dependent ER α transcriptional activity in breast cancer cells. We have found SUMO E2-conjugating enzyme Ubc9 to bind BRCA1 proteins. BRCA1 Mutant #1 (K109 to R) was impaired in its ability to both bind, as well as modulate Ubc9 mediated SUMO-dependent/independent E2-induced ER α transcriptional activity in breast cancer cells. Similarly, BRCA1 cancer -predisposing mutation (61Cys-Gly) abrogated the ability to both bind Ubc9 as well as inhibit ER α activity suggesting physiological significance. Addition of BRCA1 but not mutant #1 to E2-induced ER α in the presence of SUMO-1 and Ubc9 resulted in the degradation of ER α suggesting BRCA1 to be a putative SUMO-1 and Ubc9-dependent E3 Ubiquitin ligase for ER α . This is the first report demonstrating the participation of Ubc9 in BRCA1 E3 Ubiquitin ligase mediated degradation of ER α . These results suggest a novel function for BRCA1 in regulating the dynamic cycles of SUMO and Ubiquitin modifications required for ER α turn over and deregulation of this molecular switch due to lack of BRCA1 results in ER α -negative/positive breast cancers. This study will help in designing novel BRCA1 function-based targeted treatment for TNBC.

Probing the Microrheology of Mesenchymal Stem Cell-Based Therapeutics

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Breast cancer is a leading cause of cancer morbidity and mortality in women, afflicting 1 in 8 women world-wide and resulting in >40,000 deaths per year in the United States. Despite advances in breast cancer screening and treatment, approximately 30% of the patients presenting with early stage disease develop recurrent, advanced, or metastatic disease [1]. Current therapies, including surgery, chemotherapy, and radiotherapy, focus on treating or restraining the primary tumor and are ineffective in treating metastatic disease. The development of mesenchymal stem cell (MSC) vectors for gene delivery may offer a new method of specifically targeting breast cancer cells in tumors and their metastases and delivering prolonged levels of therapeutic proteins in the local region of the cancer cells. The development of cell-based therapeutics requires the availability of cells that can be expanded *ex vivo*, are amenable to genetic manipulation, and circulate sufficiently to allow exposure to the blood vessels and accumulation in tumors. MSCs, derived from bone marrow (BM) stroma, are multipotent progenitor cells that can self-renew and differentiate even upon *ex vivo* culture and expansion [2]. MSCs spontaneously migrate from the BM and infiltrate wounded tissues and tumors [3,4]; however, the majority of MSCs reinfused after *ex vivo* manipulation become trapped in the lungs [5]. The identification of soluble growth factors that stimulate their migration in the wound bed or tumor may be a key element in the development of MSC-based therapeutics that can overcome current transport limitations [6]. Soluble growth factors stimulate the proliferation and differentiation of MSCs *in vitro*; however, little is known about their effects on the migratory behavior of MSCs. Using multiple particle tracking microrheology we have quantified the effects of tumor secreted soluble factors on the viscoelasticity of MSCs. We have also used an *in vitro* migration assay to investigate the effect of tumor-secreted soluble factors on MSC migration. Our results indicate that treatment with tumor-secreted soluble factors increases MSC elasticity, reduces MSC viscosity, and increases MSC migration. Quantitative studies of MSC microrheology will be used to optimize the migration of MSC-based therapeutics to tumors.

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Heparan sulfate is required for cell fate commitment of embryonic stem cells

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Embryonic stem cells (ESCs) are able to self-renew infinitely and are pluripotent, *i.e.* they can differentiate into all adult cell types. The regulatory network that maintains self-renewal has been under intense investigation and is increasingly defined, however the molecular mechanisms that control the transition from self-renewal to differentiation are only poorly understood. Heparan sulfate (HS) is a linear, heavily sulfated polysaccharide that is found abundantly on the cell surface of ESCs. The HS- biosynthetic enzyme EXT1 is part of a co-polymerase complex and initiates the extension of the HS chain. In this study we investigated the role of HS by creating conditional *EXT1* knockout ESCs. Ablation of *EXT1* resulted in complete loss of HS and enabled us to study the role of HS in ESC self-renewal and cell fate commitment. *EXT1*^{-/-} ESCs could be stably maintained in long-term feeder-free culture conditions demonstrating that HS is not required for the maintenance of self-renewal. Upon reduction or complete withdrawal of LIF, an experimental condition that induces spontaneous differentiation of mouse ESCs, *EXT1*^{-/-} ESCs remained undifferentiated and failed to commit into developmental lineages. *EXT1*^{-/-} ESCs retained characteristics of pluripotent, self-renewing ESCs including high alkaline phosphatase activity, compact colony morphology and expression of pluripotency genes such as *NANOG* and *OCT-4*, revealing that HS is required for the exit of self-renewal and commitment to differentiation. Moreover, we directly show that the aberrant lineage commitment of *EXT1*^{-/-} ESCs underlies defects in FGF signaling and consequently impaired ERK1/2 activation. Heparin, a HS analogue, was able to restore ERK1/2 activity and rescued cell fate commitment. Altogether our findings identify and define HS as a novel factor involved in the initial cell fate decision of ESCs, promoting the transition from self-renewal to cell fate commitment.

Gene expression profiling analyses indicate that human ovarian surface epithelia are multipotent and capable of serving as the somatic stem cell origin of epithelial ovarian cancer.

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Background:

Accumulating evidence suggests that somatic stem cells undergo mutagenic transformation into cancer initiating cells. The serous subtype of ovarian adenocarcinoma in humans has been hypothesized to arise from at least two possible classes of progenitor cells: the ovarian surface epithelia (OSE) and/or an as yet undefined class of progenitor cells residing in the distal end of the fallopian tube. We present data that reveals OSE are somatic stem cells capable of serving as the precursor cells of ovarian cancer.

Methods:

Comparative gene expression profiling analyses were carried out on OSE removed from the surface of healthy ovaries and ovarian cancer epithelial cells (CEPI) isolated by laser capture micro-dissection (LCM). Differentially expressed genes were analyzed using gene ontology, molecular pathway, and gene set enrichment analysis algorithms. The results of the gene expression analyses were selectively confirmed using immunohistochemistry.

Results:

Consistent with multipotent capacity, genes in pathways previously associated with adult stem cell maintenance are highly expressed in ovarian surface epithelia and are not expressed or expressed at very low levels in serous ovarian adenocarcinoma. Among the over 2000 genes that are significantly differentially expressed between OSE and CEPI, a number of pathways and novel pathway interactions are defined that may contribute to ovarian adenocarcinoma development.

Conclusions:

Our results demonstrate that human ovarian surface epithelia are multipotent and capable of serving as the origin of ovarian adenocarcinoma. While our findings do not rule out the possibility that ovarian cancers may also arise from other sources, they are *inconsistent* with claims that ovarian surface epithelia cannot serve as the origin of ovarian cancer initiating cells.

Study of Machine-based Nucleus Discrimination with Nuclear Feature Quantification and Analysis Techniques for large-scale Microscopy Imaging of Diffuse Glioma

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Quantitative study of histologic features present in human tissue samples using computer image analysis techniques has recently captured intensive interests. Having emerged as a key tool not only for diagnosing diseases, but also for understanding the underlying pathological mechanisms, imaging and related machine-based image analysis methodologies have played an essential role in facilitating these goals. Motivated by the idea of alleviating the interpreters' bias and improving the diagnostic reproducibility, we explore the possibility of generating a quantitative feature set associated with the nucleus of glioma cells from digital whole-slide images of diffuse gliomas using image analysis tools. More importantly, the efficacy of derived features for characterizing and distinguishing nuclei of histologically distinct gliomas was carefully evaluated. The complete study procedure includes several sequential phases: 1) image segmentation on individual nuclei, 2) micro-anatomic feature extraction for segmented nuclei characterization, and 3) regression analysis for validating feature discriminating power. Gold standard information on virtual microscopy image data was provided by a certified surgical neuropathologist, who manually delineated boundaries of each nucleus of interest, and then graded each nucleus with an integer, predefined within a range of 1-10, representing the degree of belongingness to a specific pathological category (oligodendroglioma vs. astrocytoma). A total of 220 nuclei were randomly selected from 11 distinct regions of interest. In terms of the overall accuracy of the machine-generated nucleus segmentation, the mean ratio of the overlapped region area to the union region area recognized by human and machines reached 72.45%. The most discriminating features for describing nuclei of distinct classes were determined to be: area, eccentricity, average magnitude of gradient, and sum of Canny-edge pixels. When the best set of nuclear features was used, the mean of the Mean Absolute Grade Errors and the mean of Standard Deviation of Absolute Grade Errors associated with 100 repeated experiments having different testing (20%) and training (80%) data partitions were 1.32 and 1.23, respectively. As a result, we conclude that the system developed is promising for automating the generation of quantitative micro-anatomic features from images of glioma samples for nuclear classification.

Kinetic Model Characterization of Protease Activity in Tumor Microenvironments

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Macrophages are involved in proteolytic tissue remodeling during inflammation, and tissue associated macrophages (TAMs) are hypothesized to play roles in tumor growth and extracellular matrix breakdown leading to metastasis. The papain family of cysteine proteases, the cathepsins, is an understudied class of powerful collagenases and elastases implicated in extracellular matrix degradation that are secreted by macrophages and cancer cells and shown to be active in the slightly acidic tumor microenvironment. Due to the tight regulatory mechanisms of cathepsin activity and their instability outside of those defined spaces, detection of the active enzyme is difficult to precisely quantify, and therefore challenging to target therapeutically. Engineers use models to test hypotheses that are difficult to determine experimentally. Using valid assumptions that consider these complex interactions we have developed a system of ordinary differential equations to calculate the concentration of mature, active cathepsins in a biological space. The system of reactions considers four enzymes (cathepsins B, K, L, and S, the most studied cathepsins with reaction rates available), three substrates (collagen IV, collagen I, and elastin) and one inhibitor (cystatin C) and comprise more than 30 differential equations with over 50 specified rate constants. Along with the mathematical model development, we have been developing new ways to quantify proteolytic activity to provide further inputs. This predictive model will be a useful tool in identifying the time scale and culprits of proteolytic breakdown leading to metastasis and angiogenesis in malignant tumors.

RNA-driven DNA modifications from bacteria to human cells

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As recently demonstrated in the yeast *Saccharomyces cerevisiae* model system, RNA can be used as template for DNA synthesis by cellular DNA polymerases at the chromosomal level during the process of double-strand break (DSB) repair (Storici et al., *Nature*, 2007, 447: 338). Now we have found that the phenomenon of RNA-mediated DNA repair and modifications is not limited to yeast cells, but can also occur in mammalian cells, as well as in bacterial cells. Here we show that RNA-containing oligodeoxynucleotides can serve as templates to repair a DSB in chromosomal DNA of human cells and can introduce base modifications into the genomic DNA. We utilized RNA-containing oligodeoxynucleotides designed to repair a chromosomal break generated within a copy of the green fluorescent protein (GFP) gene randomly integrated into the human genome of HEK-293 cells. In order to test the capacity of RNA to modify DNA in bacterial cells, we examined the frequency of gene modification at the *lacZ* gene in *Escherichia coli* following transformation with RNA-containing oligodeoxynucleotides. Interestingly, the RNA-containing oligodeoxynucleotides could be used to correct a deletion mutation in the *lacZ* gene in the *E. coli* genome without any break induction, with a frequency only 5 fold less than that obtained using corresponding DNA-only molecules. These results demonstrate that RNA bases, as well as RNA molecules, can have a direct and active role in DNA modification and remodeling from bacteria to mammalian cells.

(Georgia Cancer Coalition-R9028)