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### Title

ROLE OF APE/REF-1 IN HEPATOCELLULAR CARCINOMA PROGRESSION: A CANDIDATE THERAPEUTIC TARGET?

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### S-ADENOSYLMETHIONINE STABILIZES DUAL-SPECIFICITY MAPK PHOSPHATASE PROTEIN (DUSP1) BY INHIBITING PROTEASOMAL ACTIVITY: IMPLICATION IN THE PATHOGENESIS AND CHEMOPREVENTION OF HEPATOCELLULAR CARCINOMA

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**Background & Aims:** Increased activity of mitogen-activated protein kinases (MAPKs) correlates with a more malignant phenotype in hepatocellular carcinoma (HCC). There is a reciprocal regulation between p44/42 MAPK (ERK1/2) and the dual-specificity MAPK phosphatase MKP-1/DUSP1. ERK phosphorylates DUSP1, facilitating its proteasomal degradation, while DUSP1 inhibits ERK activity. Mice deficient in methionine adenosyltransferase 1a (*Mat1a* KO) have chronic hepatic S-adenosylmethionine (SAME) deficiency, increased ERK activation, and develop HCC. Our aims were to examine whether DUSP1 expression is regulated by SAME and if so, elucidate the molecular mechanism. **Methods:** The studies were conducted using *Mat1a* KO mice livers and cultured mouse and human hepatocytes. Real-time PCR and Western blotting measured mRNA and protein levels, respectively. Proteasomal activity was measured in KO livers, cultured hepatocytes and pure 20S proteasomes. **Results:** *Dusp1* mRNA and protein levels were reduced by 45% and 50%, respectively, in 4-month old *Mat1a* KO livers. We previously showed that primary cultures of human and mouse hepatocytes rapidly lost MAT1A expression and SAME levels fell. Using this model system, we found that 12 hours after culturing, the mRNA and protein levels of DUSP1 decreased by 50% and 85%, respectively. Adding 2mM SAME to the medium significantly blunted the fall in DUSP1 protein level (40% fall) but had little effect on its mRNA level. Proteasomal chymotrypsin-like and caspase-like activities were measured and were significantly increased in *Mat1a* KO livers and hepatocytes cultured for 12 hours. SAME was as effective as proteasomal inhibitor MG132 in preventing this increase in cultured hepatocytes. Using pure 20S proteasomes, SAME inhibited chymotrypsin-like activity by 40% and caspase-like activity by 70%. The protein level of Bax, another protein degraded by the proteasome, also fell by 90% 12 hours following primary culture and this was blunted by SAME and MG132 to comparable extent. **Conclusions:** DUSP1 mRNA and protein levels are lower in *Mat1a* KO livers and fell rapidly during culture of human and mouse hepatocytes. The fall in DUSP1 protein level is partly due to increased proteasomal degradation. SAME treatment blunted the fall in DUSP1 protein level by inhibiting proteasomal activity and stabilized the DUSP1 protein to the same extent as the proteasomal inhibitor MG132. Taken together, these results suggest that SAME inhibits malignant cell growth partly by inhibiting ubiquitin-proteasomal pathway and stabilizing DUSP1. This helps to explain how SAME deficiency predisposes to HCC and effectiveness of SAME as a chemopreventive agent.

Disclosures:

The following people have nothing to disclose: Maria Lauda Tomasi, Komal Ramani, Ainhoa Iglesias-Ara, Tony W. Li, Fawzia Bardag-Gorce, Francesco Feo, Rosa M. Pascale, Jose M. Mato, Shelly C. Lu

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### THE ANTIOXIDANT TRANSCRIPTION FACTOR NF-E2-RELATED FACTOR 2 (NRF2) UP-REGULATES EXPRESSION OF THE HUMAN BILE SALT EXPORT PUMP (BSEP)

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**Background:** The bile salt export pump (BSEP, ABCB11) is a major determinant of bile formation. The BSEP gene is positively regulated by FXR and also LRH-1. Impairment of BSEP expression leads to cholestatic liver injury which may be associated with oxidative stress. The NF-E2-related factor-2 (Nrf2) is a transcription factor that responds to oxidative stress by binding to antioxidant-responsive elements (ARE) in many hepatic Phase I and II enzymes as well as hepatic efflux transporters, including MRPs. Computer transcriptional software analysis of the human BSEP gene predicts that there is a Maf recognition element (MARE) sequence in the proximal promoter region, where Nrf2 can bind. **Aim:** To determine whether the Nrf2/MARE pathway might influence the expression of BSEP. **Methods:** HepG2 cells and human hepatocytes were used to assess BSEP expression. Total mRNA was measured by TaqMan quantitative RT-PCR. Protein expression was detected by Western blotting. Promoter activity was determined by a dual luciferase reporter assay. Chromatin immunoprecipitation (ChIP) and electrophoretic mobility shift assays (EMSA) were used to identify if Nrf2 binds to the BSEP promoter. **Results:** Oltipraz, an Nrf2 activator, increased BSEP mRNA expression by 7-fold in HepG2 cells and protein by 70% in human hepatocytes. Co-transfection of Nrf2 and human BSEP promoter reporter constructs dose-dependently increased the activity of the BSEP promoter. Mutations of the predicted MARE motif completely abolished the Nrf2 transcriptional activation. ChIP and EMSA assays also demonstrated that Nrf2 specifically bound to the MARE motif in the BSEP promoter. Finally, siRNAs lowered Nrf2 expression and prevented the up-regulation of BSEP by oltipraz. **Conclusion:** These studies indicate that Nrf2 is a positive transcriptional regulator of human BSEP expression and that this effect is mediated via the Nrf2/MARE pathway. Our findings suggest that Nrf2 might regulate BSEP's expression under conditions of oxidative stress and that pharmacological activation of Nrf2 might be a therapeutic option in cholestatic liver injury.

Disclosures:

The following people have nothing to disclose: Jittima Weerachayaphorn, Shi-Ying Cai, Carol J. Soroka, James L. Boyer

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### ROLE OF APE/REF-1 IN HEPATOCELLULAR CARCINOMA PROGRESSION: A CANDIDATE THERAPEUTIC TARGET?

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Although it is well documented that elevated reactive oxygen species (ROS) and copper (Cu) are closely correlated with hepatocellular carcinoma (HCC) carcinogenesis and progression, the detailed mechanisms involved remain unclear. Apurinic/apyrimidinic endonuclease-1/redox effector factor 1 (APE1/Ref-1) is an important regulator of cellular responses to oxidative stresses. Recent studies have demonstrated that induced APE/Ref-1 facilitates tumor progression and is associated with poor prognosis. In this study, we hypothesized that APE1/Ref-1 plays a critical role in HCC progression as an important mediator of cellular proliferation, metastasis and anti-apoptosis signal pathways. Our data showed that HCC cells

(Hep3B) exhibited a higher level of APE1/Ref-1 with elevated ROS levels compared to cultured hepatocytes. Following Cu treatment, APE1/Ref-1 transcriptional activities were enhanced in hepatocytes as well as the downstream target genes (AP-1/c-Fos, Matrix Metalloproteinase-1 (MMP-1) and Bcl-2). In Hep3B cells, co-treatment with the copper chelator disulfiram inhibited the expression of AP-1/c-Fos, MMP-1 and Bcl-2. Additionally, experimental knockdown of APE/Ref-1 using siRNA in Hep3B cells resulted in decreased levels of AP-1/c-Fos, MMP-1, Snail, and Bcl-2, in parallel with corresponding alterations of proliferation, apoptosis and invasion activities. Consistently, APE/Ref-1 overexpression was accompanied with increased levels of AP-1/c-Fos, MMP-1, Snail, and Bcl-2 in hepatocytes. These results define a novel role of APE1/Ref-1 in HCC progression, and provide a basis for further investigations utilizing appropriate APE1/Ref-1 inhibitors in combination with chemodrugs for HCC treatment.

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The following people have nothing to disclose: Zhen Yang, Sun Yang, Frank L. Meyskens

### 1302 INFLAMMATORY AND ONCOGENIC SIGNALING SUPPRESS MIR-29 EXPRESSION IN CHOLANGIOCARCINOMA CELLS

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Inflammation and biliary stasis are shared features of multiple clinical conditions that increase the risk of developing of cholangiocarcinoma. Importantly, cholangiocarcinoma cells are resistant to apoptotic stimuli due to elevated expression of the prosurvival protein Mcl-1. We have previously shown that Mcl-1 is regulated by mir-29b in cholangiocarcinoma, and mir-29b expression is decreased in malignant cells. The AIM of this project was to determine if inflammatory or oncogenic signals contribute to mir-29 suppression. METHODS: The mir-29b-1~29a transcriptional unit was identified by 5' RACE and RT-PCR. Transcriptional activity of the upstream genomic sequence was investigated using luciferase promoter constructs. Expression of precursor and mature mir-29 was determined by quantitative RT-PCR. NF-kB was activated by Toll-like receptor ligands (LPS, flagellin), or bile acids (deoxycholate, taurodeoxycholate, and glycodeoxycholate). Sonic Hedgehog signaling was inhibited using cyclopamine or anti-Shh neutralizing antibody. Cells were transfected with a c-myc expression plasmid to assess the effect of myc on mir-29 expression. Non-malignant immortalized cholangiocytes (H69) and malignant cholangiocarcinoma cells (KMCH, HuCCT-1) were studied. RESULTS: Expression of mir-29b1~29a was demonstrated from a spliced primary transcript from chromosome 7 in cholangiocytes. The putative promoter (1.7 kb adjacent to the transcriptional start site) contained multiple predicted transcription factor binding sites, including NF-kB, Gli (Hedgehog signaling), and a consensus E-box myc-binding site. Promoter activity using a luciferase reporter or RT-PCR for mature mir-29b confirmed that NF-kB activators decreased mir-29 expression. Deoxycholate treatment increased Mcl-1 protein and apoptosis resistance, consistent with mir-29 mediated Mcl-1 silencing. Additionally, Hedgehog inhibition increased mir-29 promoter activity and expression dependent on the Gli-binding sequence. Finally, myc overexpression inhibited mir-29 expression and promoter activity. Surprisingly, mutation of the E-box sequence did not affect myc-mediated suppression of mir-29 promoter activity. CON-

CLUSIONS: Downregulation of mir-29 has been described in multiple cancers, though the mechanism of suppression has not been described. Here we demonstrate that signaling events important in cholangiocarcinoma pathobiology such as oncogenic signals (Hedgehog, myc), innate immune activation (TLR), and bile acids all converge to suppress mir-29 promoter activity and expression. The effect of bile acids on Mcl-1 protein expression and apoptosis resistance may be due to suppression of mir-29 expression.

Disclosures:

The following people have nothing to disclose: Justin L. Mott, Satoshi Kurita, Sophie Cazanave, Nathan Werneburg, Steven F. Bronk, Martin E. Fernandez-Zapico, Gregory Gores

### 1303 CELL-TO-CELL TRANSMISSION OF SMALL RNA BETWEEN HEPATOCYTES EXTENDS THE REACH OF RNA INTERFERENCE-BASED THERAPY FOR HEPATITIS C

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Background: RNA interference (RNAi), the degradation of cognate mRNA by small interfering RNA (siRNA), has emerged as a promising therapeutic entity for viral infections, including hepatitis C virus (HCV). In plants and invertebrates, RNAi-mediated protection can spread to neighboring cells; however, such a phenomenon has not been described in mammalian cells. In this study, we investigated whether endogenous expressed liver-specific microRNA and vector-delivered siRNA can transfer between cells and whether this exchange could extend the therapeutic effect of RNAi against HCV infection. Methods: Human hepatoma cell lines Huh7 and HepG2, Huh7-ET HCV replicon cells, and renal epithelial line 293T were co-cultured with conditioned medium or cells stably transduced with integrating lentiviral vectors expressing green fluorescent protein (GFP) and small hairpin RNAs targeting the HCV NS5b (LV-shNS5b), CD81 (LV-shCD81) or non-targeted control (LV-shCon). Liver-specific microRNA, miR-122, was quantified by real-time RT-PCR. HCV replication was determined by luciferase activity. CD81 expression was measured by flow cytometry. Results: MiR-122 is not only highly expressed in Huh7 cells but also detectable in Huh7-CM, suggesting release of miR-122 by the cells. Upon incubation of HepG2 or 293T cells, which are 200 and 50,000-fold lower in miR-122 expression, with Huh7-CM the miR-122 level in these cells was increased by 4-20 fold, indicating uptake of miR-122 from the medium. To further investigate whether small interfering RNA delivered by vectors can be transferred between cells, Huh7-ET was co-cultured with stably transfected Huh7 cells expressing shNS5b or shCon. A significant reduction of viral replication was observed at 1:1 ratio of shNS5b cells (52±12% p<0.01) but not with shCon cells. Similarly, at suboptimal transduction of Huh7 with LV-shCD81, CD81 expression of non-transduced cells was also significantly down-regulated (30±12.9%, P<0.001). Moreover, conditioned medium of transduced cells could convey RNAi-mediated silencing of HCV (39%±12, P<0.01) and CD81 (23.5%±5.1, P<0.01) without transfer of GFP, suggesting a similar mechanism of transfer as for microRNA. Conclusion: It was shown that transmission of small RNA is an innate function of human cells which can both mediate the exchange of microRNA and vector delivered RNAi, thereby extending the therapeutic effects of RNAi on HCV.

Disclosures: