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Proteomics analyses of ovarian caner using genetically defined human ovarian cancer models

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1. ABSTRACT

Using a genetically defined human ovarian cancer model, we have analyzed the protein expression profile of human ovarian cancer cells established by oncogenic RAS transformation. Cells were immortalized by retroviral transfection of SV40 t/T antigens and the catalytic subunit of telomerase (hTERT). Careful analyses of protein targets associated with oncogenic transformation have enabled us to identify several novel signaling pathways that play important roles in oncogenic transformation of human ovarian epithelial cells.

2. INTRODUCTION

Ovarian cancer is the fifth-leading cause of death among women with cancers. It is estimated that 22,430 new cases will be diagnosed in 2007, and 15,280 will die from the disease (American Cancer Society Cancer Facts and Figures, 2007). Epithelial ovarian cancer (EOC) accounts for 85-90% of ovarian tumors in adult women. Clinically EOC is the most lethal of all gynecological cancers. Several factors contribute to the poor prognosis of ovarian cancer cases. First, the disease often evades detection during its early stages due to the lack of specific symptoms and limitation in diagnostic methods. Adding to this problem, ovarian cancer frequently persists, recurs and eventually becomes resistant to conventional treatments. The single most important reason for the overall poor prognosis of ovarian cancer is the lack of a reliable and specific method for early detection. When detected early, the survival rate for ovarian cancer patients is dramatically improved. Currently, however, more than 70% ovarian cancer patients are diagnosed when the disease is advanced beyond the pelvis. The five-year survival rate in this group is approximately 15%. On the other hand, more than 90% of patients with non-metastatic ovarian cancer can be cured.

Understanding the signaling mechanisms and pathways that underlie the pathogenesis of ovarian cancer is critical to the development of more effective detection and therapeutic strategies. Recently, genetically defined models for human ovarian cancer have been established using normal human ovarian surface epithelial (HOSE) cells (24). The ability of a few discrete genetic alterations to transform human cells *in vitro* supports the concept that there may be common molecular pathways associated with the seemingly complex

and diverse phenotypes of cancer. Equally important, these model systems provide an opportunity to study the complex processes of tumorigenesis, especially the dissection of cellular signaling networks governed by individual oncogenes or tumor suppressor genes, as well as how oncogenes and tumor suppressors collaborate with each other to perturb the balance of cellular signaling networks and lead to the formation of cancer. Functional proteomics analyses of genetically defined human ovarian cancer (GDHOC) models lead to the identification of numerous protein targets and several novel cellular pathways associated with oncogenic transformation of HOSE. Our studies demonstrate that proteomics analyses of GDHOC not only provide further insight into our understanding of the mechanism of ovarian tumorigenesis transformation, but also offer a new approach for identifying potential diagnostic and therapeutic targets for EOC.

3. GENETICALLY DEFINED HUMAN OVARIAN CANCER MODELS

Animal models, especially mouse models of human cancer pathogenesis have become an important part of cancer research. Mouse models of ovarian cancer in recent years have provided new insights for understanding the biological and genetic factors important for ovarian cancer tumorigenesis and for developing potential novel strategies for diagnosis and treatments of ovarian adenocarcinomas (13). However, emerging evidence indicates that there are fundamental differences in biology between rodent and human cells, particularly in cellular processes related to oncogenic transformation. For example, long-term culture of mouse embryonic fibroblast leads to frequent spontaneous immortalization, while similar treatment of human counterpart results in cell quiescence. In addition, primary rodent cells can be readily transformed by two cooperating oncogenes (22;37), whereas the same combination of oncogenes fails to transform human primary cells. This realization of species-specific requirements for cellular transformation not only calls for caution in linking findings based on mouse cancer models directly to human tumors, but also advocates the need for establishing cancer models based on a human system. Successful isolation and culturing of pure populations of HOSE cells (4;15;43) have provided the opportunity to systematically study ovarian tumorigenesis using specific genetic manipulations.

Oncogenic transformation of human primary cells using defined genetic elements was achieved only recently (16). With the advent of this methodology, malignant transformation has been demonstrated in a variety of human cell lines using the same set of defined genetic elements. Recently, transformation of primary ovarian surface epithelial cells using oncogenes C-erbB-2 and RAS has been achieved (21;24). Both studies involve immortalization of early passage HOSE by transfecting genes containing the early region of SV40 (including both large T and small t oncogenes) and the catalytic subunit of telomerase (hTERT). While immortalized HOSEs are not tumorigenic in nude mice, expression of activated RAS or c-erbB2 in these cells results in up-regulation of VEGF (vascular endothelial growth factor) and interleukins and the development of carcinomas in nude mice (21;24). In addition, the RAS-transformed human ovarian cancer model exhibits many similar features of natural ovarian tumors (24). Most interestingly, the recently identified ovarian cancer tumor suppressor gene, OPCML (opioid binding protein/cell adhesion molecule-like gene) is also epigenetically inactivated in the RAS-transformed HOSEs (30), suggesting that the genetically defined human ovarian cancer cells also recapitulate EOC at the molecular level.

4. PROTEOMIC ANALYSIS OF GDHOC MODELS

Functional proteomics has emerged in recent years as a powerful tool for dissecting complex signaling networks and understanding biological function and regulation at the system level (11). The aforementioned GDHOC Models are perfect systems for proteomic analyses. These genetically engineered human ovarian cancer cells are not only species and tissue-specific but also genotype-specific and homogenous, and therefore amendable for comparative and reproducible protein profiling. In addition, further genetic manipulations can be performed on these models for target validation and follow-up mechanistic studies. To systematically investigate the molecular mechanisms of oncogenic RAS mediated transformation and to explore the important signaling events associated with transformation of human ovarian epithelial cells, we compared the total protein expression profiles of three genetically engineered cell lines derived from HOSE by two-dimensional gel electrophoresis (2-DE). While T29 cells, stably transfected with SV40 T/t antigens and hTERT, are fully immortalized, only the addition of oncogenic HRAS (in T29H) or KRAS (in T29K) leads to the malignant transformation of T29 cells as demonstrated by the capacity for anchorage-independent growth and tumor formation in nude mice (24). From more than 2200 resolved protein spots within the ranges of pI 4-7 and molecular weight 6,000-200,000 on silver-stained 2-DE gel, A total of 151 protein spots were identified that differ significantly between T29 and T29H cells while 130 protein spots were changed significantly between T29 and T29K cells. Interestingly, of these altered protein spots, only about 40 spots are common between T29H and T29K (Figure 1).

Using peptide mass fingerprinting, we successfully identified 55 of these altered protein spots (52 unique proteins). The apparent observed molecular mass and pI for all identified proteins matched very well with their calculated values. Protein targets identified in our 2-DE proteomic analysis are involved in multiple cellular processes such as growth/proliferation; metabolism/energy production; apoptosis/cell death; cellular signaling; cellular transport; adhesion/movement; cytoskeletal organization/morphology; cell division/replication; protein biosynthesis, repair, folding, degradation; antioxidant functions; transcription/translation, mRNA splicing, DNA repair; and calcium signaling. These proteins are distributed in different cellular localizations and many of them assume multiple cellular functions (Table 1). The largest group of proteins identified consists of enzymes involved with metabolic processes. This is not surprising since tumor cells often demand higher metabolic levels to maintain a growth advantage over normal cells and this finding is consistent with the notion of increased energy demand in cancer cells (3). A significant portion of proteins identified have been implicated in disease processes. For example,

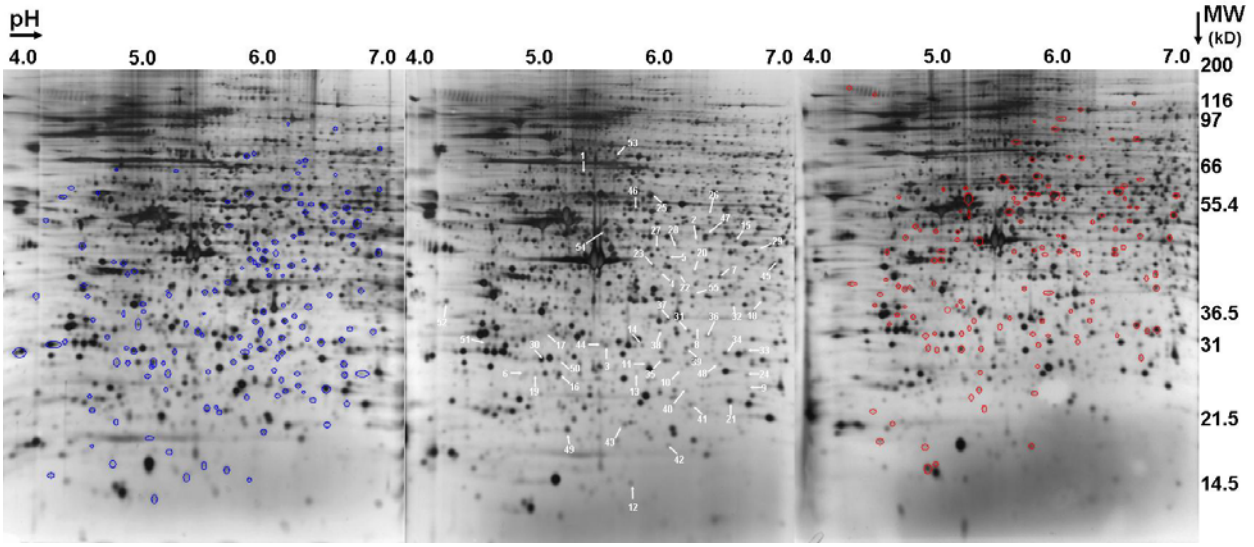

Figure 1. Proteomic analysis of T29, T29H, and T29K cells using 2-DE gels. Whole cell lysates (200 μg) from T29H (A), T29 (B), and T29K (C) cells were separated on 2-DE gels and visualized by silver staining. Protein spots significantly altered between T29 and T29H cells (A) or between T29 and T29K cells (C) are highlighted in blue and red, respectively. Arrows indicate identified protein spots significantly altered in T29H or T29K cells (B).

aldehyde dehydrogenase 2, ornithine aminotransferase, enoyl-CoA hydratase (33), and the calcium dependent protease Calpain 1, have been previously shown to associate with cancer development. Furthermore, calretinin, a mediator of intracellular calcium responses (36), cathepsin D, and glyoxalase I are biomarkers used in diagnosis of cancer, including ovarian epithelial tumors (8) (17). Increased levels of cathepsin D are associated with poor prognosis in breast and ovarian cancer patients (25) and RAS is known to up-regulate cathepsin D activity in human breast epithelial cell lines (7). The alteration of these known cancer associated proteins and biomarkers further supports our genetically defined human cancer cell model as an appropriate mimic of naturally occurring cancers *in vivo*. The ability to identify these biomarkers and cancer-related proteins also validates our approaches in studying the mechanism of oncogenic transformation. More importantly, we have identified many novel protein targets that have been not previously associated with RAS signaling/transformation. These proteins present a unique opportunity to delineate new pathways that might be involved in the transformation process mediated by oncogene RAS.

To further dissect the signaling network involved in RAS mediated transformation, we analyzed our proteomic results using Ingenuity Pathways Analysis (IPA, Ingenuity® Systems (http://www.ingenuity.com/products/pathways_analysis.html). These analyses revealed three major networks. The first network involves extracellular growth factors TGF-beta-1 (transforming growth factor beta 1) and KIT ligand, intracellular signaling molecules AKT1 and RAC1, and the nuclear transcription factor MYC (Figure 2A). The second network includes key signaling molecules such as hepatocyte nuclear factor 4α (HNF4α), WNT1 (wingless-type MMTV integration site family, member 1), SIM (single-minded homolog 1), TP73L (tumor protein p73-like), RAS and p21 (Figure 2B). The ability to use the identified protein targets to trace back to the RAS oncogene, the original cause of oncogenic transformation, provides an independent validation for our approaches and results. The third network contains TGFα (transforming growth factor α), PTEN (phosphatase and tensin homolog), RELA (v-rel reticuloendotheliosis viral oncogene homolog A), MYB (v-myb myelobastosis viral oncogene homolog), MAP3K5 (mitogen-activated protein kinase kinase kinase 5), YWHAZ (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide) and VHL (von Hippel-Lindau tumor suppressor) (Figure 2C). These molecules are important for many cellular processes such as growth, proliferation, apoptosis, survival, differentiation, cell cycle progression and transformation. Most of them have been implicated in tumorigenesis. In addition, there is extensive cross-talking among these signaling networks based on published literature (Figure 2). These pathway analyses reveal that RAS pathway activation induces oncogenic transformation through interactions with multiple oncogene and tumor suppressor pathways in ovarian cancer cells.

5. NOVEL ANTI-APOPTOTIC PATHWAYS MEDIATED BY RAS ONCOGENE IN GDHOC

While expression of $HRAS^{V12}$ or $KRAS^{V12}$ leads to oncogenic transformation of immortalized HOSE, the growth rates of T29H and T29K are not significantly different from that of the parental cell, T29. These results suggest that the oncogenic transforming ability of oncogene RAS is not primarily duo to growth/proliferation stimulation. We, therefore, hypothesized that RAS may exert its oncogenic effect through inhibiting cell death or promoting cell survival. One apparent candidate related to apoptosis and cell survival is a member of the caspase family, procaspase 4. This pro-

Table 1. Identifications and functions of proteins identified to be changed significantly between T29 and T29H/T29K cells

Protein Name	NCBI Acc #	Subcellular Location	Function													
			G/P	M/EP	A/CD	CS	CT	A/M	CO	M	CD/R	PB	DI	AF	T/T	Ca
Proteins Commonly Changed																
L-plastin variant	Q59GX5	C,M	*					*	*	*						
Caspase 4 isoform alpha precursor	NP_001216	C,ER,M			*			*	*	*		*				
Cathepsin D preproprotein	NP_001900	PM,C,ER,GV, V	*		*			*			*	*	*			
Calponin 3	NP_001830	C,M,.PM							*							
Selenophosphate synthetase	NP_036379	U		*												
Human Glyoxylase I	NP_006699	C		*	*									*		
Gelsolin-like capping Protein	NP_001738	C,N						*	*	*						
Nicotinate-nucleotide pyrophosphorylase	Q15274	U		*									*			
Peroxiredoxin 3 isoform b	NP_054817	C,M,N	*	*	*									*		
Guanidinoacetate N-methyltransferase isoform a	NP_000147	C		*									*			
NADH dehydrogenase ubiquinone	NP_004542	C,M		*												
Phosphohistidine Phosphatase 1	NP_054891	C				*										
Phosphoserine phosphatase	NP_004568	U		*									*			
Latexin	AAH05346	C		*		*										
Tumor susceptibility gene 101	NP_0062283	PM,C,GV,N	*		*	*				*	*	*	*		*	
Glyoxalase I	NP_006699	C		*	*									*		
Calretinin (calbindin 2)	NP_001731	C														*
Serine/threonine phosphatase 1 gamma	BAA82664	C,PM,N	*			*		*			*					
Homo Sapiens 14q32 jagged gene	AAD15563	C		*												
Proteins solely changed in T29H																
Calponin 3	NP_001830	C,MC,PM							*							
Peroxiredoxin 3	NP_006784	C,M,N	*	*	*									*		
THO complex 3 (THOC3)	NP_115737	N													*	
SPFH domain family,member 2 isofrom 1	NP_009106	PM														
Protein-L-isoaspartate O-methyltransferase	NP_005380	C		*								*				
Copine I	NP_003906	U					*									*
Aldehyde dehydrogenase, mitochondrial precursor	P05091	C,M		*	*									*		
Mannose-6-phosphate isomerase	NP_002426	C		*									*			
CGI-17 protein	AAH22789	C									*	*				
Omithine aminotransferase, mitochondria precursor	PO4181	C,M		*									*			
Calpain, small subunit I	NP_001740	C, ER, GV, PM	*		*	*		*					*			*
3-hydroxyisobutyrate dehydrogenase	NP_689953	C,M		*										*		
AASDPP transferase	NP_056238	C		*								*	*			
Delta 3, delta 2-enoyl-CoA isomerase	AAA35485	C,M		*												
Mito. Short-chain enoyl-CoA hydratase 1 precursor	NP_004083	C,M		*												
Thioredoxin peroxidase	NP_006397	C	*			*								*		
PEF	AAQ89370	C														*
Replication protein A2, 32 kDa	NP_002937	C,N													*	
S-phase protein	AAP97193	U		*	*	*										
6-phosphogluconolactonase	NP_036220	C		*												
DNA directed RNA polymerase II polypeptide E	NP_002686	PM,N		*		*							*		*	
Chain A, Human Dj-1 with sulfinic acid	1SOAA	C,M,N	*		*	*							*			
Mago-Nashi homolog	NP_002361	C,N					*								*	
Stathmin	NP_005554	C	*		*	*		*	*		*					
Cathepsin D preproprotein	NP_001900	PM,C,ER,GV	*		*						*	*	*			
Proteins solely changed in T29K																
Methionine adenosyltransferase II, alpha	NP_005902	C		*												
Vimentin	NP_003371	PM,C,MC,N	*					*	*	*						
Calcium-binding transporter	AAF28888	U			*											*
Similar to GrpE protein homolog 1, mito. Precursor	XP_00112752 2	C,M										*				
Eukaryotic translation initation factor 5A	NP_001961	C,N	*		*										*	
Rho GDP dissociation inhibitor (GDI) alpha	NP_004300	C			*		*	*	*	*	*					
14.3.3 protein	AAC28640	C,N			*	*	*				*					
Clathrin, light polypeptide A, isoform a	NP_001824	N,PM				*	*		*							
Keratin 1	NP_006112	PM,C,N						*	*							*
Cytokeratin 18	CAA21277	C,MC,PM,N			*				*	*						
Isocitrate dehydrogenase 3 (NAD+) alpha precursor	NP_005521	C,M		*										*		

Abbreviations: Subcellular Location Abbreviations: C - Cytoplasm; ER - Endoplasmic Reticulum; GV - Golgi Vesicles; M - Mitochondria; MC - Microfilaments/Microtubule; N - Nucleus; PM - Plasma Membrane; V- Vesicles; U – Unknown, Function Categories: G/P - Growth/Proliferation; M/EP - Metabolism/Energy Production; A/CD - Apoptosis/Cell Death; CS - Cellular Signaling; CT - Cellular Transport; A/M - Adhesion/Movement; CO - Cytoskeletal Organization; M - Morphology; CD/R - Cell Division/Replication; PB - Protein Biosynthesis, Repair, Folding, Degradation; DI - Disease Involvement; AF - Antioxidant Functions; T/T - Transcription/Translation, mRNA splicing, and DNA repair; Ca - Calcium Signaling, * denotes the presence of the specific cellular function.

apoptotic protease is up-regulated in the RAS-transformed cells (48). The increased level of procaspase 4 was further confirmed by immunoblotting analysis using caspase 4 specific antibodies. Up-regulation of a caspase during RAS-mediated oncogenic transformation is surprising since we expect that oncogene RAS would suppress cellular apoptotic pathways. However, when caspase 4 activity was measured, we observed that the cellular activity of caspase 4 was significantly lower in RAS transformed HOSE as compared to that of the immortalized parental cells. Since the active state of caspase 4 consists of a tetramer of P20 and P10 subunits derived from the proteolytic processing of procaspase 4 and given the fact that while levels of the procaspase 4 were increased, overall basal cellular caspase 4 activity was actually decreased in RAS transformed HOSE, we concluded that the maturation of procaspase 4 was blocked at the post-translational level in RAS-transformed T29H cells. The fact that up-regulation of procaspase 4 could be reversed by U0126, a MEK specific inhibitor, demonstrated that this inhibitory effect on caspase 4 maturation was directly mediated by the MAP kinase pathway down-stream of RAS. This inhibitory mechanism could be due to a phosphorylation modification of procaspase 4 by MAP kinase, in a manner similar to procaspase 9 whose maturation has been shown to be blocked by HRAS activation *via* phosphorylation at Thr125 (1). The exact mechanism of RAS-mediated inhibition of caspase 4 activation is currently under further investigation.

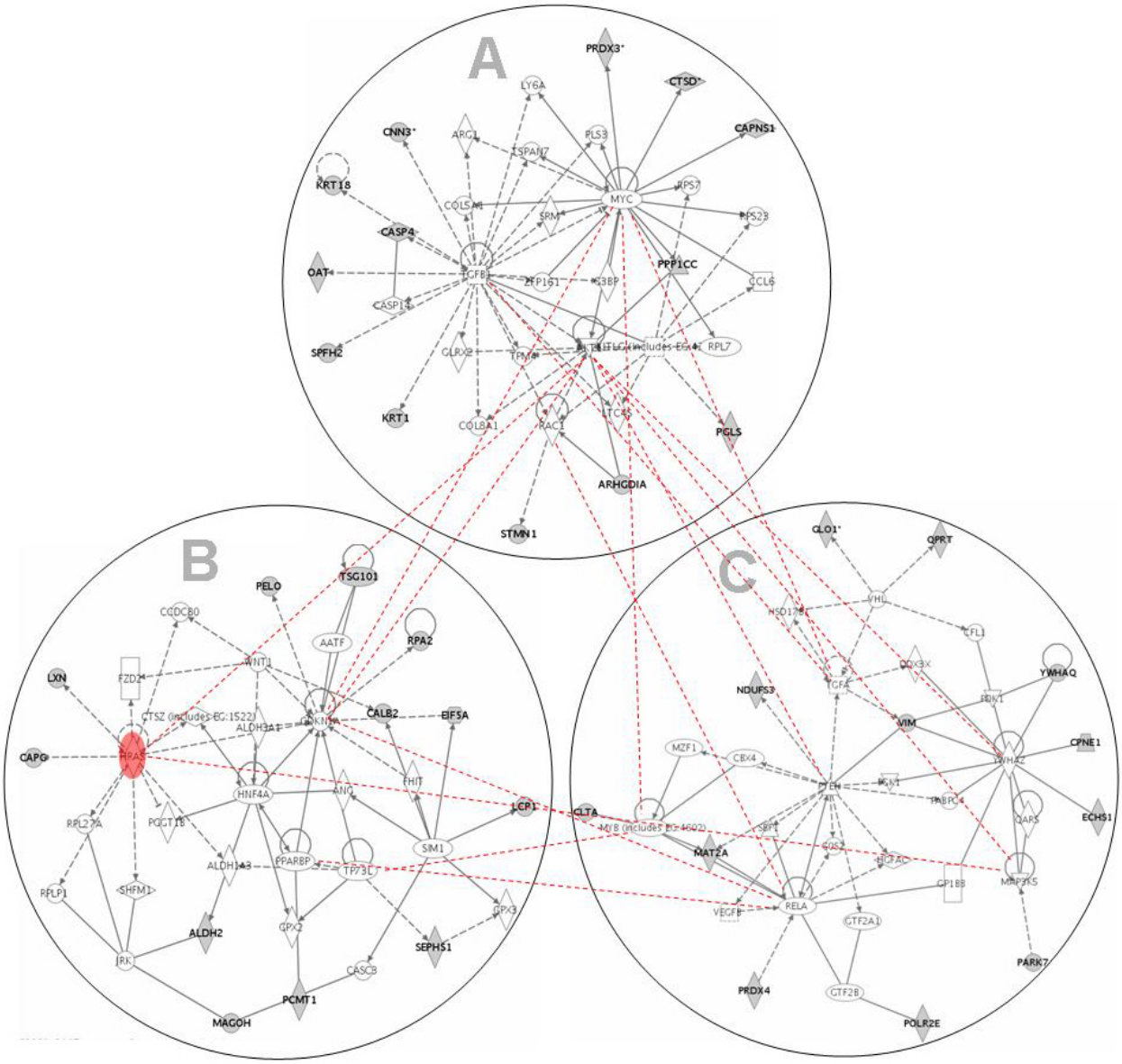

Figure 2. Major signaling networks involved in RAS mediated transformation. Proteomic results were analyzed using Ingenuity Pathways Analysis (IPA, Ingenuity® Systems (http://www.ingenuity.com/products/pathways_analysis.html). Protein targets identified to be significantly altered between T29H and T29 or T29K and T29 are highlighted in gray. Red dashed lines represent major cross-talks among signaling networks.

Caspase 4 activation has been implicated in Fas-mediated apoptosis (18;27). The apparent increased level of procaspase 4 protein and decreased caspase 4 activity in T29H cells led us to hypothesize that oncogenic RAS may be protecting the transformed T29H cells from Fas-mediated apoptosis by suppressing the maturation of caspase 4 and/or other related proteases involved in cellular apoptosis. To test this hypothesis, we subjected the T29 and T29H cells to an anti-Fas antibody CH11 (Upstate Biotechnology) that cross-links the death receptor Fas and leads to the formation of a death-inducing signaling complex. Treatment of CH11 antibody led to massive cell death and significant apoptosis indicated by the activation of caspase 3 in T29 cells, while the transformed T29H cells were not significantly affected by CH11 treatments under the same conditions. When cell lysates from T29 and T29H cells treated with CH11 antibody were probed with a caspase 4 specific antibody, activation of caspase 4 as monitored by the formation of active caspase 4 P20 and P10 subunits was observed in T29 cells, while significantly less P20 and P10 subunits and a concomitant increase in caspase 4 intermediates were detected in T29H cells (48). These results further confirmed that RAS mediated oncogenic transformation blocked the maturation of caspase 4 and attenuated Fas-mediated apoptosis in human ovarian surface epithelial cells. The apparent blockage of caspase 4 activation and resistance to Fas-mediated apoptosis suggests that RAS-mediated suppression of caspase 4 activation may allow HRAS transformed cells to evade apoptosis.

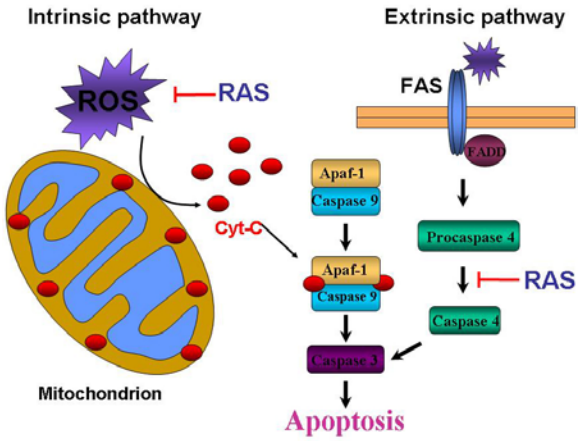

Figure 3. Suppression of major cellular apoptotic pathways by oncogene RAS. Our proteomics studies demonstrate that activation of RAS signaling pathways up-regulates major cellular antioxidant pathways, which suppress the intrinsic apoptotic pathway. On the other hand, RAS can also inhibit the activation of the extrinsic apoptotic pathway through blocking the maturation of procaspase 4.

A large portion of the proteomic changes associated with RAS-mediated transformation involves proteins important in cellular metabolism and redox balance (48). Several are enzymes involved either directly in metabolizing reactive oxygen species (ROS) or in maintaining the redox balance of the cell. These proteins include thioredoxin peroxidase (peroxiredoxin 4), a thiol-specific antioxidant enzyme that uses thioredoxin as a source of reducing equivalents to scavenge hydrogen peroxide; peroxiredoxin 3, a mitochondrial member of the antioxidant family of thioredoxin peroxidases; NADH dehydrogenase ubiquinone Fe/S protein, a 30 kDa subunit of mitochondrial complex I of the electron transport chain; glyoxyalase I, a key enzyme in detoxification of the reactive dicarbonyl compound, methylglyoxal, a side product of glycolysis; and selenophosphate synthetase, a rate-limiting enzyme for incorporating selenium into key redox metabolizing enzymes, such as glutathione peroxidase and thioredoxin reductase. Up-regulation of these antioxidant proteins protected RAS-transformed T29H cells from H_2O_2-mediated cell killing and suppressed the H_2O_2-mediated caspase 3 activation in T29H cells. In addition, suppressing RAS oncogene activity using retrovirus-based shRNA specifically against $HRAS^{V12}$ re-sensitized T29H cells to H_2O_2-mediated cell killing (50). These results suggest that RAS oncogene is directly responsible for the observed up-regulation of cellular antioxidation capacity and consequently protects transformed cells from oxidative-stress related cell death. Such an enhanced antioxidation capability may constitute a common mechanism for tumor cells to evade apoptosis induced by oxidative stresses at the higher ROS loads that have been observed in cancer cells.

Our proteomic studies of GDHOC have revealed that activation of the RAS/MAPK signaling pathways lead to the suppression of two major cellular apoptotic pathways in ovarian cancer. On one hand, RAS suppresses the extrinsic apoptosis pathway mediated by the death receptor through inhibition of the activation of procaspase 4. On the other hand, RAS up-regulates important cellular antioxidation pathways, which in turn protect mitochondria against ROS-mediated damages and prevent the initiation of intrinsic apoptotic pathway (Figure 3).

6. UP-REGULATION OF TUMOR SUSCEPTIBILITY GENE 101 (TSG101) PROMOTES CELL GROWTH AND SURVIVAL

TSG101 is a protein significantly up-regulated post-transcriptionally following transformation of human ovarian surface epithelial cells with oncogenic HRAS or KRAS (49). To our knowledge, this is the first report linking RAS signaling and regulation of TSG101 protein levels. TSG101 was initially cloned as a stathmin-interacting protein in a yeast two-hybrid screen (28). TSG101 is a multifunctional protein. TSG101 is an integral component of the endosomal sorting complex I required for transport, ESCRT-I (39). The N-terminal region of TSG101 shares extensive sequence homology to the Ubc domain of ubiquitin-conjugating enzyme but lacks a critical active-site cysteine essential for enzymatic activity. The Ubc domain allows TSG101 to specifically recognize mono-ubiquitinylated proteins and mediate endosomal trafficking important for membrane receptor endocytosis and retroviral budding (2;5;6;10;14;26;31;41). In addition, TSG101 contains a putative DNA-binding motif at its C-terminus and can act as a transcriptional cofactor to repress or activate nuclear hormone receptor-mediated transactivation (44).

Previous studies show that the primary mechanism for TSG101 protein regulation occurs at the level of protein degradation (12), and TSG101 and MDM2 form an autoregulatory loop that modulates the cellular levels of both proteins (23). To understand the molecular mechanism of RAS-mediated TSG101 regulation, we examined the roles that $p14^{ARF}$ and HDM2, two RAS down-stream targets, played in controlling TSG101 cellular levels. Our study demonstrates that oncogenic RAS, acting through the RAF/MEK/ERK signaling cascade as shown previously (32), up-regulates $p14^{ARF}$, which in turn suppresses cellular activity of HDM2 and consequently leads to the increase of cellular TSG101 protein levels in RAS-transformed cells (49).

To determine the significance of TSG101 up-regulation and the role that TSG101 may play in tumor formation and development in human ovarian cancer, we used siRNA to knock down TSG101 in the ovarian carcinoma cell line SKOV-3, which lack a mutated RAS gene, but displays hyperactivated RAS signaling pathways similar to the engineered T29H cells (45). In addition to growth inhibition, TSG101 knockdown cells exhibited a significant increase of cells in G_2/M phase and a decrease in G_1/G_0 phase cells when compared to control siRNA transfected cells by flow cytometric cell cycle analyses, indicating a blockage in the cell cycle at G_2/M phase. A significant population of sub-G1 apoptotic cell debris was also observed in TSG101 knockdown cells. These observations suggest that silencing of TSG101 results in G_2/M arrest and subsequent cell death in SKOV-3 cells. When injected into athymic nude mice, SKOV-3 cells with TSG101 silenced induced significantly smaller tumors *in vivo*, suggesting that TSG101 has a definitive prosurvival effect on this ovarian epithelial cancer cell line (49). These

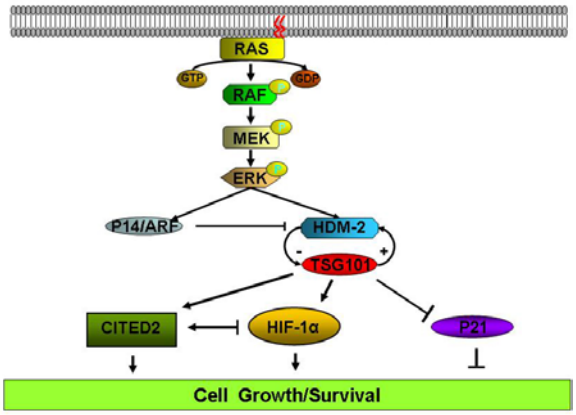

Figure 4. Mechanism of RAS-mediated TSG101 regulation. Activation of the RAS/RAF/MEK/MAP kinase leads to transcriptional induction of $p14^{ARF}$ that suppresses cellular HDM2 activity (35). Inactivation of HDM2 leads to elevated cellular levels of TSG101 through a negative-feedback loop (23). Increased TSG101 levels in ovarian cancer cells promote cell growth and survival by suppressing p21 expression and enhancing CITED2/HIF-1α expression and activation.

findings are in agreement with recent data pointing to a more growth promoting rather than tumor suppressive effect of TSG101 as proposed earlier (9;20;52) and suggest that RAS-mediated up-regulation of TSG101 is a positive factor for cell survival and tumor growth in ovarian cancer cells.

To determine the molecular mechanism of TSG101 knockdown-mediated inhibition of cell viability and tumorigenicity, we checked the expression of a panel of genes involved in controlling cell cycle progression and cell survival. Suppression of TSG101 expression in SKOV-3 cells is accompanied by the reduction of two closely related transcriptional factors; CBP/p300-interacting transactivator with ED-rich tail 2 (CITED2) and hypoxia inducible factor 1α (HIF-1α) (49). Both CITED2 and HIF-1α play important roles in the regulation of cell growth and survival. HIF-1α controls the expression of more than 70 genes (29), plays a critical role in cancer cell survival (38) and tumor migration and metastasis (34;46). *CITED2* knockout in mice is embryonic lethal and *CITED2*$^{-/-}$ mouse embryonic fibroblasts senescence prematurely and express increased levels of the cell proliferation inhibitors $p16^{INK4a}$, $p19^{ARF}$, and $p15^{INK4b}$, suggesting that CITED2 is essential for cell proliferation and survival (19;47). On the other hand, over expression of CITED2 in Rat1 cells led to anchorage-independent growth in soft agar and tumor formation in nude mice (40). In addition to their individual roles, HIF-1α and CITED2, through a common partner, CBP/p300, have also been shown to operate in a negative feedback loop to control oxygen homeostasis. Under low oxygen condition during hypoxia, HIF-1α activates the transcription of CITED2. Accumulation of CITED2 inhibits HIF-1α transactivation by blocking its interaction with CBP/p300 and consequently attenuates hypoxia responses (42). Because of the important roles that CITED2 and HIF-1α play in cell proliferation and survival, the apparent growth inhibitory and apoptotic effects of TSG101 gene silencing observed in the SKOV-3 cells can be explained at least in part by the loss of CITED2/HIF-1α expression.

In addition to the decreased levels of CITED2 and HIF-1α, the expression of one important tumor suppressor, the cyclin-dependent kinase inhibitor p21 is significantly up-regulated both at the mRNA and protein levels in SKOV-3 cells with reduced TSG101. Using a luciferase reporter gene under the transcriptional control of the p21 promoter and a chromatin immunoprecipitation (ChIP) assay, we further demonstrate the recruitment of TSG101 to the p21 promoter where TSG101 acts as co-repressor of p21 transcription in ovarian cancer cells. Interestingly, this negative correlation between TSG101 and p21 can also be observed in human ovarian tumor samples using tumor microtissue arrays (51). Taken together our study demonstrates a novel mechanism in which TSG101 promotes cell survival and proliferation through suppression of p21 and concomitant up-regulation of CITED2 and HIF-1α (Figure 4).

7. OVER-EXPRESSION OF TSG101 IN OVARIAN CANCER SAMPLES AND ITS ROLE IN PATIENT SURVIVAL

To determine the clinical significance of the TSG101 up-regulation observed in our GDHOC model, we monitored the expression levels of TSG101 in epithelial ovarian cancer (EOC) using human ovarian cancer tissue arrays. While normal human ovarian surface epithelium did not show significant expression for TSG101, the expression of TSG101 was increasingly positive in borderline tumors, low grade and high grade carcinomas compared to normal HOSE. The levels of expression for TSG101 varied among ovarian carcinomas showing a wide range of variability from totally negative (26.3%) to strongly positive (23%) with over 70% of the samples showing certain degrees of TSG101 up-regulation. TSG101 was most frequently up-regulated in serous carcinoma, poorly differentiated carcinomas and malignant mixed mullerian tumors as opposed to the other histotypes. The levels of TSG101 expression in EOC positively correlated with the grade and stage of the cancer. Patients with grade 3 ovarian cancers showed a significantly higher percentage of high TSG101 as compared with patients with grade 1 or grade 2 and patients with stage 3 or stage 4 had a significantly higher percentage of high TSG101 as compared with patients with stage 1 or stage 2. Furthermore, this increased TSG101 concentration is associated with poor prognostic outcomes. About 33% of ovarian cancer patients with high expression of TSG101 survive more than 5 years while the 5-year survival rate for patients with low expression of TSG101 is about 53%.

8. PERSPECTIVE

This study is the first analysis of proteomics of a GDHOC model. The power of this approach rests on the fact that a large number of cellular targets associated with oncogenic transformation can be identified and studied simultaneously. This not only leads to the identification of novel signaling targets but also allows a systematic analysis of a group of proteins with similar cellular functions that otherwise would not be possible. As shown in the case with the up-regulation of a large number of antioxidant enzymes, our proteomics analyses suggest that an enhanced antioxidant capacity protects the transformed cells from high levels of ROS associated with the uncontrolled growth potential of tumor cells. An enhanced antioxidation capability may

constitute a common mechanism for tumor cells to evade cell death by suppressing the intrinsic apoptotic pathway induced by oxidative stresses. In parallel, we have also shown RAS-mediated oncogenic transformation suppresses the extrinsic apoptotic pathway by blocking the maturation of caspases initiated by the death receptor at the cell membrane. Finally, our study reveals an unexpected and novel mechanism of TSG101 regulation through the RAS signaling pathway. RAS-mediated up regulation of TSG101 provides pro-growth/survival stimuli through the suppression of the cell-cycle inhibitor p21 and up-regulation of pro-survival factors HIF-1α and CITED2. Most importantly, the discovery of TSG101 up-regulation in genetically defined human ovarian cancer models leads us to further demonstrate that TSG101 is overexpressed in more than 70% ovarian carcinomas and may represent a potential target for therapeutic intervention.

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Abbreviations: 2-DE: two-dimensional electrophoresis; CITED2: CBP/p300-interacting transactivator with ED-rich tail 2; EOC; ovarian epithelial cancer; GDHOC: genetically defined human ovarian cancer cell; HIF-1α: hypoxia inducible factor 1α; HMD2: human homolog of MDM2; HOSE: human ovarian surface epithelia; hTERT: catalytic subunit of human telomerase; MDM2: mouse double minute 2; ROS: reactive oxygen species; TSG101: tumor susceptibility gene 101.

Key Words: Ovarian Cancer; Proteomics, Transformation, RAS, Tumor Susceptibility Gene 101, Procaspase 4, p21, HIF-1α, CITED2, Review

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