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Defects in Cell Growth Regulation by $C_{18:0}$-Ceramide and Longevity Assurance Gene 1 (LAG1) in Human Head and Neck Squamous Cell Carcinomas (HNSCC)#

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Running title: LAG1 and C_{18}-ceramide in the regulation of growth of HNSCC

ABSTRACT

In this study, endogenous long chain ceramides were measured in 32 human head and neck squamous cell carcinoma (HNSCC) and 10 non-squamous head and neck carcinoma tumor tissues, as compared to adjacent non-cancerous tissues, by liquid chromatography/mass spectroscopy (LC/MS). Interestingly, only one specific ceramide, $C_{18:0}$ ceramide, was selectively down regulated in the majority of HNSCC tumor tissues. On the other hand, in non-squamous tumor tissues, this selectivity for C_{18}-ceramide was not detected. These data suggested the hypotheses that decreased levels of C_{18}-ceramide might impart a growth advantage to HNSCC cells, and that increased generation of C_{18}-ceramide may be involved in the inhibition of growth. These roles were examined by reconstitution of C_{18}-ceramide at physiologically relevant concentrations in UM-SCC-22A cells (SCC of hypopharynx) via overexpression of mammalian upstream regulator of growth and differentiation factor 1 (mUOG1), a mouse homologue of longevity assurance gene 1 (mLAG1), which has been shown to specifically induce the generation of C_{18}-ceramide. LC/MS analysis showed that overexpression of the mLAG1/mUOG1 resulted in increased levels of only $C_{18:0}$-ceramide by about 2-fold, i.e. concentrations similar to those of normal head and neck tissues. Importantly, increased generation of C_{18}-ceramide by mLAG1/mUOG1 inhibited cell growth (about 70-80%), which mechanistically involved the modulation of telomerase activity, and induction of apoptotic cell death by mitochondrial dysfunction. In conclusion, this study demonstrates, for the first time, a biological role for LAG1 and C_{18}-ceramide in the regulation of growth of HNSCC.

INTRODUCTION

Squamous cell carcinoma of the head and neck (HNSCC), one of the six most common cancers in the world, is associated with poor survival and high mortality rates. Global occurrence rate of HNSCC is estimated to be about 5% of the total malignancies in the adult population, and in the United States there are about 40,000 annual cases of HNSCC (1-3). Despite advances in treatment, including surgery, radiation and chemotherapy, survival statistics of patients with this disease have not improved significantly in decades (3,4). Although p53, cyclin D1, K-ras, Rb and telomerase have been identified as prognostic markers for HNSCC (5), delineation of pathogenic mechanisms is required for understanding the biology of these tumors, designing optimized therapy, and for identification of specific diagnostic and prognostic markers.

The bioactive sphingolipid ceramide, an emerging tumor suppressor lipid, mediates anti-proliferative responses such as apoptosis, cell cycle arrest and senescence (6,7). Our recent studies have shown that ceramide is one of the upstream regulators of telomerase activity (8-10), and that the regulation of telomerase by ceramide involves the inactivation of c-Myc transcription factor via increased ubiquitin/proteasome function for its rapid proteolysis (11). It is well established that telomerase is active in about 80-90% of the tumor tissues of the HNSCCs, whereas it is not active in normal head and neck tissues (12-15). These results suggested to us that endogenous levels of ceramide may also be mis-regulated in HNSCC.

Endogenous ceramide levels can be altered by various mechanisms, including de novo synthesis of ceramide or activation of sphingomyelinases, which can regulate cell growth (16). One of the key enzymes of the *de novo* pathway is (dihydro)ceramide synthase, and in *Saccharomyces cerevisiae* longevity assurance gene 1 (Lag1) and its homologue Lac1 were identified as components of ceramide synthase activity (17, 18). Recent studies have shown that one of the mouse homologues of LAG1, known as upstream of growth and differentiation factor 1 (mUOG1 or mLAG1) selectively regulates the synthesis of stearoyl (C_{18})-containing sphingolipids, including C_{18}-ceramide (19). The human homologue of the mLAG1/mUOG1 has recently been identified (hLAG1, LAG1Hs or LASS1), and has >80% amino acid homology with mLAG1 (20, 21). Subsequently, two additional homologues of mammalian Lag1 family members, *trh1* and *trh4*, were identified to regulate ceramide synthesis using different fatty acyl CoA donors (22). Overexpression of *trh1* increased mainly stearic acid and arachidic acid-containing sphingolipids, and *trh4* overexpression elevated mainly palmitic acid-containing sphingolipids (22).

Since the clinical relevance of endogenous ceramide and its roles in the regulation of cell growth in HNSCC has not been established previously, this study first focused on examining the levels of endogenous ceramide in clinical samples obtained from patients with HNSCC. Unexpectedly, the data demonstrated that only C_{18}-ceramide is down regulated significantly in the majority of the HNSCC when compared to their adjacent normal tissues. However, in non-squamous tumors, this selective down-regulation of C_{18}-ceramide was not detected, suggesting a specific role for C_{18}-ceramide in the

pathogenesis/progression of HNSCC. Further evidence is presented to establish the role of LAG1/C_{18}-ceramide in the inhibition of growth, modulation of telomerase activity and induction of apoptosis in HNSCC cells in vitro. To our knowledge, this is the first study demonstrating a biological role for LAG1 and C_{18}-ceramide in the regulation of growth of HNSCC.

EXPERIMENTAL PROCEDURES

Tissue samples and statistical analysis. Tumor and their paired adjacent non-cancerous tissues of HNSCC and non-squamous head and neck cancer patients were obtained from the Tumor Bank at the Hollings Cancer Center (Medical University of South Carolina) with the permission of the Institutional Review Board (IRB). Normal-adjacent tissues, which were mucosal tissues that were not grossly or histologically cancerous or pre-cancerous, were excised at least 1 cm away from the main tumor mass of the patients. Some of the patients (HNSCC patients #1-17) have tissues from their tumor mass and from their normal-adjacent part, which were referred as "paired" samples, and some patients (HNSCC patients # 18-32) have only their tumor tissues without normal adjacent tissues. The type of disease and the levels of C_{18}-ceramide in tumors as compared to normal tissues are summarized in Table I and II. Ceramide measurements in these samples were performed by LC/MS as described below. Statistical analysis of the results were performed using student t-test.

Measurement of ceramide levels using high performance liquid chromatography/mass spectrometry (LC/MS). The cellular levels of endogenous ceramides were measured using normal phase high performance liquid chromatography coupled to atmospheric pressure chemical ionization-mass spectrometry (LC/MS) as described previously (9). Ceramide levels were normalized to total protein levels (0.5 mg protein/sample).

Cell lines and culture conditions. Human head and neck cancer cell lines UM-SCC-1 (SCC of retromolar trigone/floor of the mouth) and UM-SCC-22A (SCC of

hypopharynx) cells (23) were obtained from Dr. Thomas Carey at the Department of Otolaryngology/Head and Neck Surgery, University of Michigan. Cells were grown in DMEM containing 10% FCS and 1% penicillin/streptomycin at 37^{o}C in 5% CO_2.

Detection of growth inhibition by MTT and trypan blue assays. The concentrations of agents that inhibited cell growth by 50% (IC_{50}) were determined from cell survival plots obtained by MTT or trypan blue assays as described (8). Triplicate wells were used for each treatment.

Plasmids and transfections. The complete cDNA (1.4 kb) for mammalian UOG1 (mLAG1/mUOG1) was cloned in the pCMVexSVneo plasmid and used for transfections of UM-SCC-22A and UM-SCC-1 cells using Effectine Transfection Kit (Qiagen) as described previously (8).

Quantitative Real-time PCR (Q-PCR) and conventional semi-quantitative reverse-transcription PCR (RT-PCR). One μg of total RNA, isolated using an RNA isolation kit (Qiagen), was used in reverse transcription reactions as described (8). The resulting total cDNA was then used in the Q-PCR or RT-PCR as described previously (16, 8). The mRNA levels of β-actin and rRNA were used as internal controls.

Measurement of telomerase activity. Telomerase activity in cell extracts was measured by the PCR-based telomere repeat amplification protocol (TRAP) using TRAPeze kit

(Intergen, Gaithersburg, MD) which includes a 36-bp internal control to allow quantification of activity as described (8-10).

Analysis of mitochondrial membrane potential. The collapse of an electrochemical gradient across the mitochondrial membrane during apoptosis was measured using JC-1 Mitochondrial Membrane Potential Detection Kit (Cell Technology) by flow cytometry as described by the manufacturer. This kit uses a unique cationic dye, JC-1, to signal the loss of the mitochondrial membrane potential. In healthy cells, the dye accumulates in the mitochondria as aggregates, which become fluorescent red. In apoptotic cells, the mitochondrial potential collapses, the JC-1 cannot accumulate within the mitochondria and remains in the cytoplasm as a green fluorescent monomeric form. These different forms of JC-1 were then detected by flow cytometry as described by manufacturer.

RESULTS

Analysis of ceramide levels in HNSCC tumor tissues. The levels of endogenous ceramides in 17 pairs of HNSCC, and 10 pairs of non-squamous head and neck tumor tissues were measured, and compared to their paired adjacent non-cancerous tissues using LC/MS as described. Unexpectedly, as shown in Fig 1A, the results showed that the total ceramide levels were higher in the HNSCC tumor tissues as compared to their non-cancerous tissues (2393 and 1505 pmole/0.5 mg protein, respectively). However, when individual ceramide species were examined in these paired samples (n=17), the results demonstrated that only $C_{18:0}$-ceramide was approximately 50% lower in HNSCC tumor tissues as compared to their non-cancerous counterparts (103 versus 196 pmole/0.5 mg protein) whereas the levels of all the other ceramides were generally higher in HNSCC tumors than their controls (Fig. 1B). When the levels of $C_{18:0}$-ceramide were further evaluated in each patient sample, the results showed that 12 out of 17 patients (70% of the patients, $p<0.01$) had significantly lower levels of $C_{18:0}$-ceramide in their tumor tissues compared to their adjacent non-cancerous tissues (Fig. 2A, and Table 1, left panel).

To investigate whether decreased levels of C_{18}-ceramide is due to altered expression of hLAG1, a human homologue of mLAG1/mUOG1, which has been shown to specifically increase C_{18}-ceramide generation, total RNA was isolated initially from normal and HNSCC tumor tissues of the patient #6, and then mRNA levels of hLAG1 were examined by RT-PCR and Q-PCR (Figs 2B and C, respectively). The results showed that hLAG1 mRNA expression was significantly decreased in the tumor as compared to its

adjacent normal tissue (Figs. 2B, lanes 3 and 2, respectively, and 2C), and this correlated with the decreased levels of C_{18}-ceramide in the tumor tissue of this patient (see Fig. 2A). However, mRNA levels of hLAG1 were similar in both the normal and tumor tissues of patient #9 (Fig. 2D), whose C_{18}-ceramide levels were also comparable in these tissues. These data indicate that C_{18}-ceramide levels might be regulated by the expression of LAG1 at the mRNA level in HNSCC, however, this needs to be determined in a larger cohort of patient samples, when they are available.

The levels of $C_{18:0}$-ceramide in non-cancerous tissues obtained from patients with HNSCC were similar (about 11.5 pmole/500 μg protein/sample with a standard deviation of +/- 4). Therefore, the average level of $C_{18:0}$-ceramide of these non-cancerous tissues was used in the examination of ceramide levels in 15 additional HNSCC tumor tissues for whom matched non-cancerous tissues were not available (Fig. 3A, and Table 1, right panel). The results of the LC/MS analysis showed that 7 out of 15 tumor tissues exhibited significantly lower levels of $C_{18:0}$-ceramide as compared to controls (Fig. 3A and Table 1, right panel). Taken together, these results demonstrate that $C_{18:0}$-ceramide levels are significantly lower in about 19 out of 32 (about 60% of the patients, $p<0.01$) HNSCC tumor tissues (see Table 1). In addition, the level of $C_{18:0}$-ceramide in the serum of HNSCC patients were similar to that of their non-cancerous head and neck tissues (the average value of $C_{18:0}$-ceramide in the serum of these patients was about 10.5 pmole/500 μg protein), suggesting that the amount of this ceramide in the tumor site, and not in the whole blood/serum, may be important for its regulatory roles in the pathogenesis/progression of the HNSCC.

Interestingly, in non-squamous tissues (n=10), a selective decrease in the levels of C_{18}-ceramide was not detected. Instead, lower levels of C_{16}- and C_{18}-ceramides (lower in 6 out of 10 patients, with a $p<0.005$), were observed in the tumor tissues as compared to their adjacent non-cancerous tissues (Fig. 3B and C, respectively, and Table 2). Similar results were also observed in non-squamous cell lung carcinomas, in which significantly reduced levels of C_{16}-, C_{18}- and C_{24}-ceramides were detected in the majority of the tumors (about 80%) as compared to their adjacent normal lung tissues (n=10, $p<0.001$, data not shown).

Taken together, these results suggest an important and novel role for C_{18}-ceramide and LAG1 in the pathogenesis/progression of the HNSCC whereas, in non-squamous tumors (head and neck, and lung), decreased levels of major ceramides such as C_{16}-, C_{18}- and C_{24}-ceramides appear to be important.

Analysis of the role of $C_{18:0}$-ceramide by overexpression of the mLAG1/mUOG1 cDNA in the regulation of cell growth in UM-SCC-22A cells. Decreased levels of $C_{18:0}$-ceramide in the majority of the HNSCC tumor tissues compared to their adjacent non-cancerous tissues suggested the hypothesis that while altered levels of C_{18}-ceramide might play important roles in the pathogenesis/progression of HNSCC, its increased generation/accumulation might regulate the growth of HNSCC cells. To test this hypothesis, mLAG1/mUOG1 was overexpressed in the human HNSCC cell line UM-SCC-22A (SCC of the hypopharynx) as described in Experimental Procedures. Overexpression of mLAG1/mUOG1 was confirmed by Q-PCR (Fig. 4A). This resulted in

an increase selectively in the levels of C_{18}-ceramide from about 6 to 11.5 pmol/0.5 mg protein (Fig. 4B), which are similar to the levels detected in normal head and neck tissues (Fig. 2A). Interestingly, the levels of other major ceramides such as C_{14}-, C_{24}-, and dihydro-C_{16}-ceramides (Fig. 4C, left panel) and C_{16}-ceramide (Fig. 4C, right panel) were decreased significantly, when compared to vector transfected controls.

The effects of mLAG1/mUOG1 on the regulation of growth, telomerase activity, and apoptosis. The effects of overexpression of mLAG1/mUOG1 on cell growth were examined using the trypan blue exclusion assay. The results demonstrated that mLAG1/mUOG1 expression caused approximately 82% inhibition of cell growth as compared to controls (Fig. 5A). Similar results were also obtained using MTT assays, in which overexpression of mLAG1/mUOG1 resulted in about 80% decrease in cell growth in these cells (data not shown).

Since ceramide is known to mediate the inhibition of telomerase in various human cancer cells (8, 9) and since telomerase activity has been detected in the majority of HNSCC tumors and not in normal head and neck tissues (12-15), the role of C_{18}-ceramide in the inhibition of telomerase was examined in UM-SCC-22A cells. The data showed that increased generation of C_{18}-ceramide by mLAG1/mUOG1 resulted in a significant inhibition of telomerase activity (about 50%) when compared to controls (Fig. 5B, lanes 2 and 1, respectively). Interestingly, additional data using Q-PCR indicated that the inhibition of telomerase activity by LAG1/C_{18}-ceramide may not be due to decreased mRNA expression of its catalytic reverse transcriptase (hTERT) (shown in Fig. 5C) or

RNA (hTR) subunits (data not shown), suggesting a post-transcriptional regulation. In addition, analysis of cell cycle by flow cytometry showed that mLAG1/mUOG1 significantly increased the number of apoptotic cells in the sub-G0/G1, whereas there were no significant changes in the cell cycle profiles (Fig. 5D). To further evaluate whether mLAG1/mUOG1/C_{18}-ceramide-induced apoptosis involves the mitochondrial death pathway, analysis of mitochondrial potential using JC-1 by flow cytometry was performed as described in Experimental Procedures. The data showed that mLAG1/mUOG1 expression resulted in a significant loss of mitochondrial membrane potential, as determined by increased accumulation of JC-1 (about 39%) as green monomers in the cytoplasm (Fig. 5E), suggesting a role for LAG1/C_{18}-ceramide in mediating the mitochondrial death pathway.

To determine whether the role of increased generation of $C_{18:0}$-ceramide by mLAG1/mUOG1 in the inhibition of growth is cell line specific, mLAG1/mUOG1 was expressed in another human HNSCC cancer cell line, UM-SCC-1 (SCC of the floor of the mouth), and the levels of ceramides and its effects on growth were determined using LC/MS and MTT assays, respectively. Expression of mLAG1/mUOG1, confirmed by RT-PCR (Fig. 6A, right panel), resulted in a significant increase (about 6-fold) in the generation of $C_{18:0}$-ceramide (Fig. 6A and B), whereas the levels of other ceramides, especially C_{16}-ceramide, were significantly reduced as compared to controls (Fig. 6B, upper and lower panels). Increased levels of $C_{18:0}$-ceramide in response to mLAG1/mUOG1 overexpression were also accompanied by a significant inhibition of cell growth (around 70%) in these cell lines (Fig. 6C). Thus, these results strongly

demonstrate that inhibition of growth by mLAG1/mUOG1 and $C_{18:0}$-ceramide is not cell line specific, but rather can be detected in other human HNSCC cell lines.

DISCUSSION

The results presented in this study demonstrate that decreased levels of $C_{18:0}$-ceramide might play a role in the pathogenesis/progression of the HNSCC, on the other hand, in non-squamous tissues, this selectivity for C_{18}-ceramide was not detected. These results suggest that decreased levels of C_{18}-ceramide may impart a growth advantage to cancer cells, whereas increased generation/accumulation of C_{18}-ceramide may lead to inhibition of growth of HNSCC cells. This was further supported by the data showing that increased generation of $C_{18:0}$-ceramide via overexpression of mLAG1/mUOG1 results in the inhibition of growth, which involves the modulation of telomerase activity and induction of apoptosis in HNSCC cells. Taken together, these results provide evidence for the role of LAG1 and C_{18}-ceramide in the regulation of telomerase and apoptosis in HNSCC cells.

The anti-proliferative roles of endogenous ceramide have been demonstrated in various human cancer cells previously (reviewed in 7). A recent study demonstrated that total ceramide levels are inversely correlated with malignant progression and poor prognosis of astrocytomas (24). The results from the present study support these conclusions, and they further provide additional information that the levels of specific ceramide species, such as C_{18}-ceramide, and not total ceramide levels, might also be important in the pathogenesis and regulation of cell growth in some carcinomas such as HNSCC. Therefore, the identification of immediate down-stream targets of C_{18}-ceramide, which are involved in the inhibition of cell growth in HNSCC cells, is extremely important, and of a great interest in our laboratory. In fact, the present study shows that overexpression

of mLAG1/mUOG1 results in the inhibition of telomerase activity, which has been shown to be elevated in the majority of HNSCC tumor tissues and not in the normal tissues (12-15). Thus, these results support that telomerase is one of the cancer specific targets of endogenous ceramide. Interestingly, inhibition of telomerase in response to C_6-ceramide or daunorubicin was linked to the generation of endogenous C_{16}- and C_{24}-ceramides in the A549 human lung adenocarcinoma cells (8). This inhibition, however, was mainly due to decreased mRNA expression of hTERT, and correlated with cell cycle arrest, but not apoptosis. However, in HNSCC cells, the inhibition of telomerase activity by LAG1/C_{18}-ceramide pathway appears to be at the post-transcriptional level, and correlates with apoptosis. Thus, these data suggest that specific ceramides might have distinct functions in the regulation of telomerase and apoptosis, and these functions might be cell type or tissue specific.

Longevity assurance gene 1 (LAG1) was discovered in yeast, and it has been shown that deletion of LAG1 extends life span in yeast (17, 25, 26). The mammalian homologues of yeast LAG1 were later identified in mice, called mammalian upstream of growth and differentiation 1, mUOG1 (19), and in human called human LAG1 (also named as LASS1) (17, 25). The mUOG1 and LASS1 (which have been referred to as mLAG1/mUOG1 and hLAG1, respectively in this study) have been shown to have >80% amino acid homology (20). Further studies showed that the biological activities of LAG-member genes encode essential components of acyl-CoA-dependent (dihydro)-ceramide synthase (18). Interestingly, mUOG1 has been shown to be involved in the synthesis of C_{18} (stearic acid) containing sphingolipids, including $C_{18:0}$-ceramide (19). However, the

role for these LAG-member proteins in the regulation of cell growth in mammals has not been shown previously. Therefore, our results presented here demonstrate that LAG1/C_{18}-ceramide, is involved in the regulation of cell growth in UM-SCC-22A and UM-SCC-1 cells.

Interestingly, our data demonstrated that the levels of C_{16}-ceramide is significantly up-regulated in HNSCC tumors as compared to their normal adjacent tissues. In parallel with these findings, the level of C_{16}-ceramide is greatly reduced upon mLAG1/mUOG1 over-expression and increased generation of C_{18}-ceramide in HNSCC cells (see Figs. 4 and 6). These results are surprising, because the role for increased C_{16}-ceramide in apoptosis upon induction by IgM via de novo pathway in Jurkat cells, or in cell cycle arrest and the inhibition of telomerase activity in the A549 non-squamous lung cancer cells, have been demonstrated previously (27, 10). Therefore, determining the possible relationship, if any, between increased levels of C_{16}-ceramide in HNSCC tumors, and its decreased levels in response to mLAG1/mUOG1 expression in HNSCC cell lines, is important and need to be further explored. In light of this, specific down-stream targets and sub-cellular localization/compartmentalization of these ceramides (C_{16}- and C_{18}-ceramides), might be involved in their distinct functions for the regulation of growth or apoptosis in various different types of cancer cells, and this is of a great interest in our laboratories.

It is also known that increased ceramide generation in response to various stress stimuli including radiation and chemotherapeutic agents can result in the inhibition of growth of various cancer cells (28-30). Treatment of the Tu138 human head and neck squamous

cell carcinoma cells with paclitaxel and C_6-ceramide in combination synergistically inhibited cell growth (31), demonstrating a role for ceramide in the treatment of head and neck cancer cells in vitro. However, clinical relevance of ceramide in HNSCC is still unknown. The results also indicate that the use of LC/MS (9, 32, 33) which can identify the levels of different ceramide species in a given sample is a powerful tool to discover the roles for specific ceramide species (with different fatty acid chain length) in the pathogenesis of various carcinomas including HNSCC. In fact, metabolomic profiling of sphingolipids in human glioma cell lines have been performed previously (34). The results have shown that different glioma cell lines had differences in the amounts and types of sphingolipids, and the fatty acyl chain distributions of sphingolipids were also different among these cell lines (34). It is also interesting that our results specifically showed decreased levels of C_{18}-ceramide selectively in the squamous cell carcinoma of the head and neck tissues whereas lower levels of C_{16}- and C_{18}-ceramides were detected in non-squamous head and neck tumor tissues. The association of lower C_{18}-ceramides, if any, with prognosis and/or survival in these patients with HNSCC could not be examined due to high grades of the tumors, and the lack of follow up information of the patients for a longer period of time. The clinical importance of C_{18}-ceramide in overall survival and prognosis in HNSCC needs to be determined in studies with a larger cohort of patients.

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TABLE I

Alterations of C_{18}-ceramide levels in human HNSCC tumor tissues

Ceramide levels in HNSCC tumor tissues were measured by LC/MS. For patients 1-17 C_{18}-ceramide levels are shown as percent change compared to their normal adjacent tissues, or for patients 18-32 they were compared to the average ceramide levels in normal tissues of patients 1-17. Results are means of at least two independent measurements, and standard deviations are shown in parentheses. Statistical analysis was performed using student t test, and $p<0.05$ was considered significant. Patients with significantly reduced ceramide are shown in bold. FOM, floor of the mouth.

Patient #	Type of SCC	% C_{18}-ceramide	Patient #	Type of SCC	% C_{18}-ceramide
1	Tongue	158 (5.1)	18	Oral cavity	**11** (1.8)
2	Oral cavity	**66** (1.7)	19	Oral cavity	67 (3)
3	Oral cavity	**9** (0.3)	20	Larynx	**46** (2.7)
4	FOM	207 (7)	21	Larynx	60 (4)
5	FOM	**20** (0.6)	22	Neck	**35** (1)
6	Tonsil	**30** (0.8)	23	Neck	**25** (1.8)
7	Oral cavity	**2** (0.2)	24	Hypopharynx	**46** (2.1)
8	Larynx	**7** (0.3)	25	FOM	**25** (2)
9	Cheek	**88** (2.9)	26	Tongue	86 (3)
10	Neck	**12** (0.5)	27	Tongue	69 (3)
11	Tongue	**23** (1.2)	28	Oropharynx	67 (3.6)
12	Cheek	112 (4.5)	29	Hypopharynx	70 (4.5)
13	Tongue	**54** (2.2)	30	Tonsil-tongue	84 (4.8)
14	FOM	**18** (0.7)	31	Tongue	174 (18)
15	Tongue	**67** (1.8)	32	Oropharynx	**36** (6.4)
16	Oral cavity	110 (3.4)	Normal (average of 1-17)		100 (36)
17	Tongue	**74** (4.6)			

TABLE II

The alterations of C_{16}- and C_{18}-ceramides in non-squamous head and neck cancer tumors

Ceramide levels in non-squamous head and neck tumor tissues were measured by LC/MS. Ceramide levels are shown as percent change compared to that of their paired normal adjacent tissues. Results are means of at least two independent measurements, and standard deviations are shown in parentheses. Statistical analysis was performed using student t test, and $p<0.05$ was considered significant.. Patients with significantly reduced ceramide are shown in bold. PC/A, papillary carcinoma/adenoma; PA, pleomorphic adenoma; OS, osteosarcoma; GN, ganglioneuroma; and CCC, clear cell carcinoma.

Patient #	Type of disease	% C_{16}-ceramide	% C_{18}-ceramide
1	Thyroid (PC/A)	**18** (4.3)	**27** (3.6)
2	Thyroid (PC/A)	**28** (12)	**38** (5)
3	Thyroid (PC/A)	1206 (130)	710 (50)
4	Parotid (PA)	156 (13)	91 (10)
5	Parotid (PA)	**14** (2.7)	**12** (1.9)
6	Parotid (PA)	1516 (387)	655 (227)
7	Mandible (OS)	1990 (105)	740 (185)
8	Neck (GN)	**36** (6)	**28** (8)
9	Maxilla (CCC)	**5** (1.5)	**11** (1.8)
10	Oral cavity (CCC)	**38** (4.3)	**45** (11)

FIGURE LEGENDS

FIG. 1. **Analysis of ceramide levels in tumor versus normal tissues of patients with HNSCC.** Levels of ceramide in tumors and their paired adjacent normal tissues obtained from patients with HNSCC were measured by LC/MS as described in Materials and Methods. First, total ceramide levels (A) and ceramide species (B) in 17 paired samples of HNSCC tissues were analyzed. The total levels of only C_{18}-ceramide was lower in tumor as compared to normal tissues (B). Error bars represent standard deviations, and when not seen, they are smaller than the thickness of the lines on graphs.

FIG. 2. **The levels of C_{18}-ceramide in HNSCC tumors as compared to their adjacent normal counterparts.** Levels of C_{18}-ceramide in 17 paired (normal versus tumor) tissues obtained from HNSCC patients were examined by LC/MS (A). The expression levels of hLAG1 mRNA in tumor (T) and normal (N) tissues obtained from patient #6 was measured using RT-PCR (B) or Q-PCR (C), and from patient #9 using Q-PCR (D) . Lane 1 in B contains molecular weight markers. Statistical analysis of the data was done using student t test, and $P<0.005$ was considered significant. Error bars represent standard deviations, and when not seen, they are smaller than the thickness of the lines on graphs.

FIG. 3. **Analysis of the levels of ceramides in HNSCC and non-squamous head and neck cancer tissues.** Levels of C_{18}-ceramide was measured in 15 additional HNSCC tissues (patients # 18-32), which did not have paired normal adjacent tissues, and compared to the average levels of C_{18}-ceramide in normal tissues of patients 1-17 (A). In addition, the levels of C_{16}- and C_{18}-ceramides were measured in 10 tumor patients with

non-squamous head and neck carcinomas and compared with that of their paired adjacent normal tissues (B and C, respectively). Statistical analysis of the data was done using student t test, and $P<0.005$ was considered significant. Error bars represent standard deviations, and when not seen, they are smaller than the thickness of the lines on graphs.

FIG. 4. **The role of increased generation of C_{18}-ceramide by mLAG1/mUOG1 in the inhibition of growth in UM-SCC-22A cells.** The expression of mLAG1/mUOG1 after 48 hr transfection was confirmed by Q-PCR (A). Then, the levels of C_{18}-ceramide (B) and other major ceramide species (C, left and right panels) were analyzed by LC/MS. The results shown are representative of at least two independent experiments. Error bars represent standard deviations, and when not seen, they are smaller than the thickness of the lines on graphs.

FIG. 5. **Effects of mLAG1/mUOG1 on the regulation of growth, telomerase activity, and apoptosis.** The effects of increased C_{18}-ceramide via overexpression of mLAG1/mUOG1 after transient transfections for 48 hr on cell growth was detected by trypan blue studies (A). Telomerase activity was determined by the TRAP assay as described in Experimental Procedures (B). The 36-bp non-telomerase product was used as an internal control (IC) in the TRAP assay kit. The quantification of telomerase activity was done as described by the manufacturer using the Quantity One (BioRad) software. The mRNA levels of hTERT (C) was measured by Q-PCR, and the levels were normalized to that of beta-actin. Cell cycle profiles and apoptotic cell populations (sub G0/G1) were detected by flow cytometry as described in Experimental Procedures (D).

Mitochondrial membrane potential was measured using the JC-1 kit by flow cytometry (E) as described in Experimental Procedures. The results shown are representative of at least two independent experiments. Error bars represent standard deviations.

FIG. 6. **The role of mLAG1/mUOG1 in the inhibition of growth in UM-SCC-1 cells.** The role of C_{18}-ceramide in the inhibition of growth was examined by overexpression of mLAG1/mUOG1 in UM-SCC-1 cells. The overexpression of mLAG1/mUOG1 after transient transfections for 48 hr (lane 2) were confirmed by RT-PCR (A, right panel), which resulted in increased generation of only C_{18}-ceramide (A and B), and not other ceramide species (B, upper and lower panels). The effects of increased C_{18}-ceramide on the inhibition of cell growth via expression of mLAG1/mUOG1 (C) was determined using MTT assays. The results shown are representative of two independent experiments. Error bars represent standard deviations, and when not seen, they are smaller than the thickness of the lines on graphs.

Figure 1

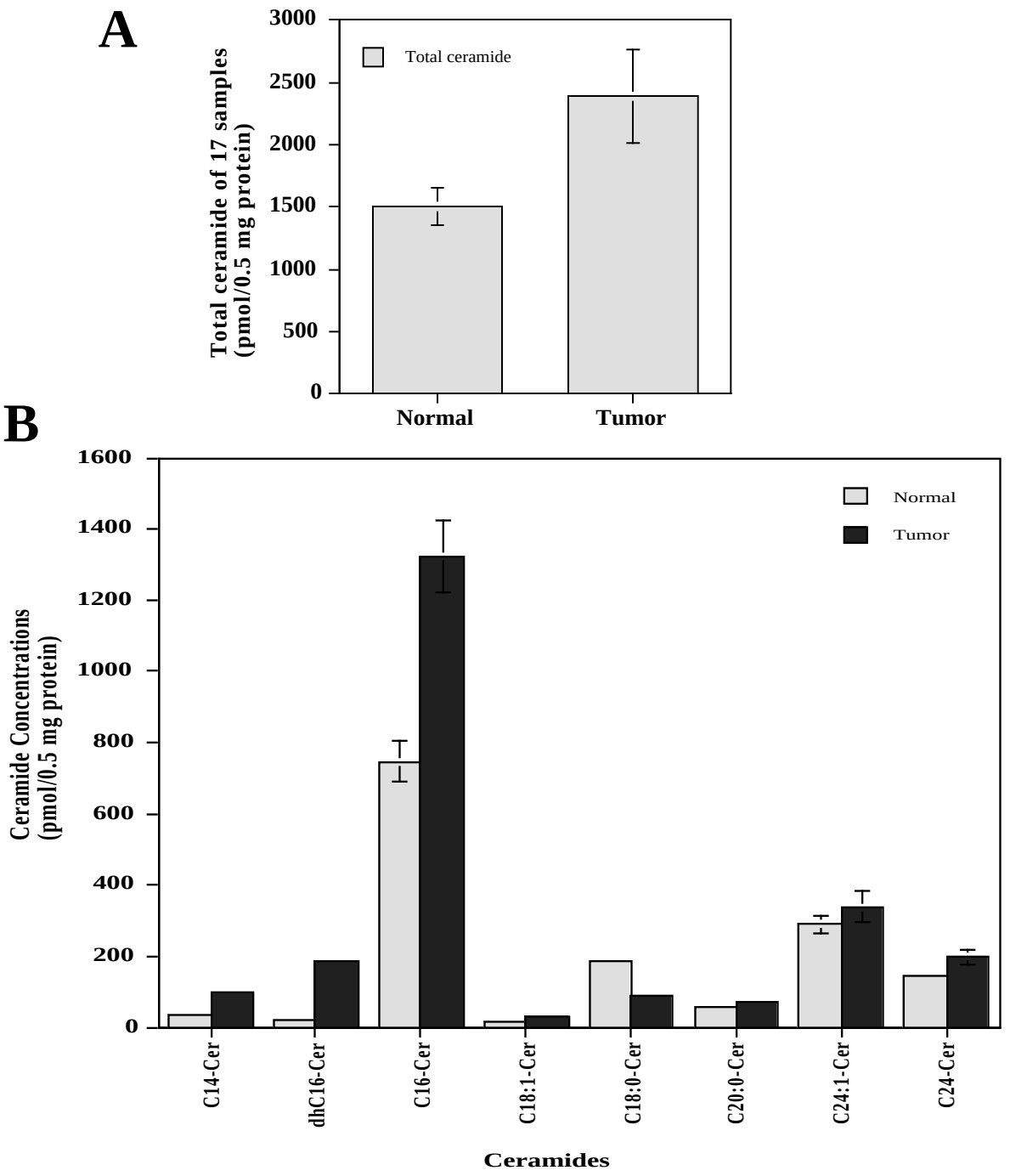

A
Total ceramide
Total ceramide of 17 samples (pmol/0.5 mg protein)
3000
2500
2000
1500
1000
500
0
Normal
Tumor
B
Normal
Tumor
Ceramide Concentrations (pmol/0.5 mg protein)
1600
1400
1200
1000
800
600
400
200
0
C14-Cer
dhC16-Cer
C16-Cer
C18:1-Cer
C18:0-Cer
C20:0-Cer
C24:1-Cer
C24-Cer
Ceramides

Figure 2

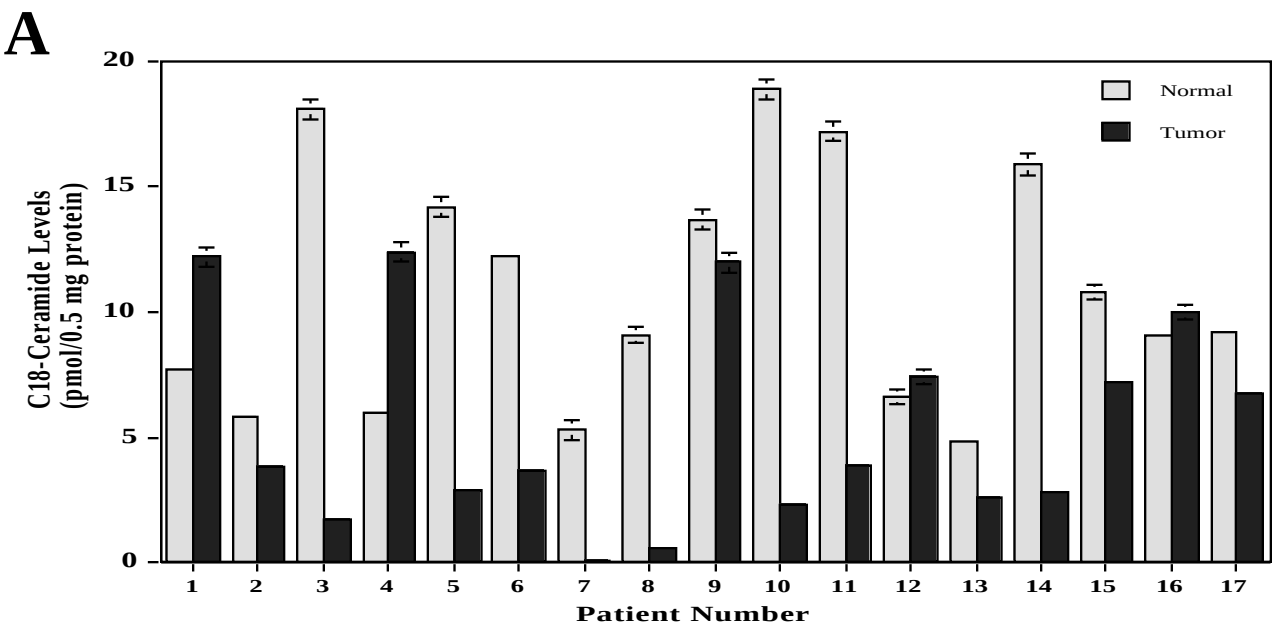

A
C18-Ceramide Levels (pmol/0.5 mg protein)
Normal
Tumor
Patient Number
B
1 2 3 4 5
hLAG1
beta-actin
N T N T
Patient #6
C
% hLAG1 mRNA Expression (Normalized to beta-actin)
LAG1 expression
N
Patient #6
T
D
% hLAG1 mRNA Expression (Normalized to beta-actin)
hLAG1
N
T
Patient #9

Figure 3

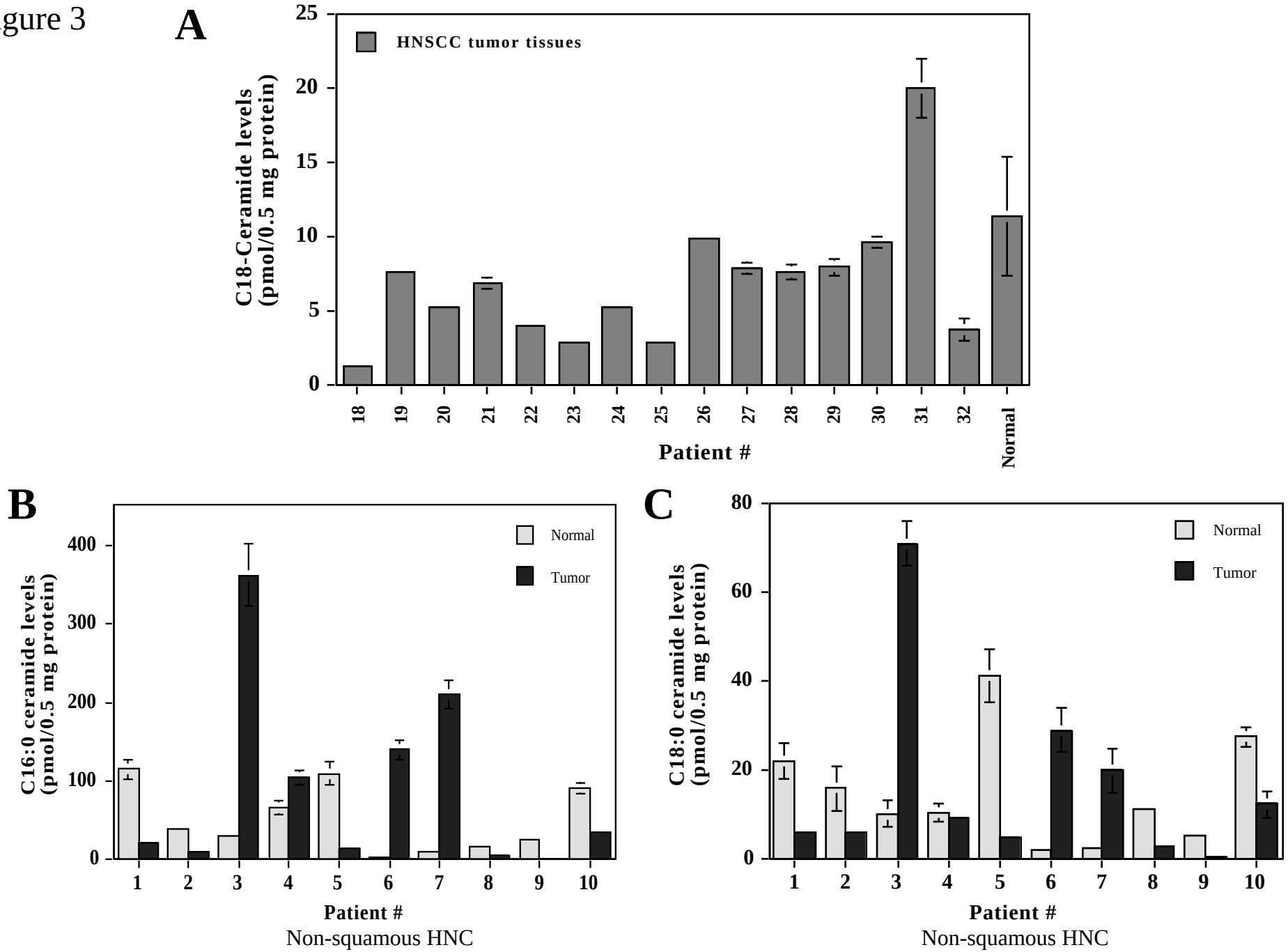

A
HNSCC tumor tissues
C18-Ceramide levels (pmol/0.5 mg protein)
0
5
10
15
20
25
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
Normal
Patient #
B
Normal
Tumor
C16:0 ceramide levels (pmol/0.5 mg protein)
0
100
200
300
400
1
2
3
4
5
6
7
8
9
10
Patient #
Non-squamous HNC
C
Normal
Tumor
C18:0 ceramide levels (pmol/0.5 mg protein)
0
20
40
60
80
1
2
3
4
5
6
7
8
9
10
Patient #
Non-squamous HNC

Figure 4

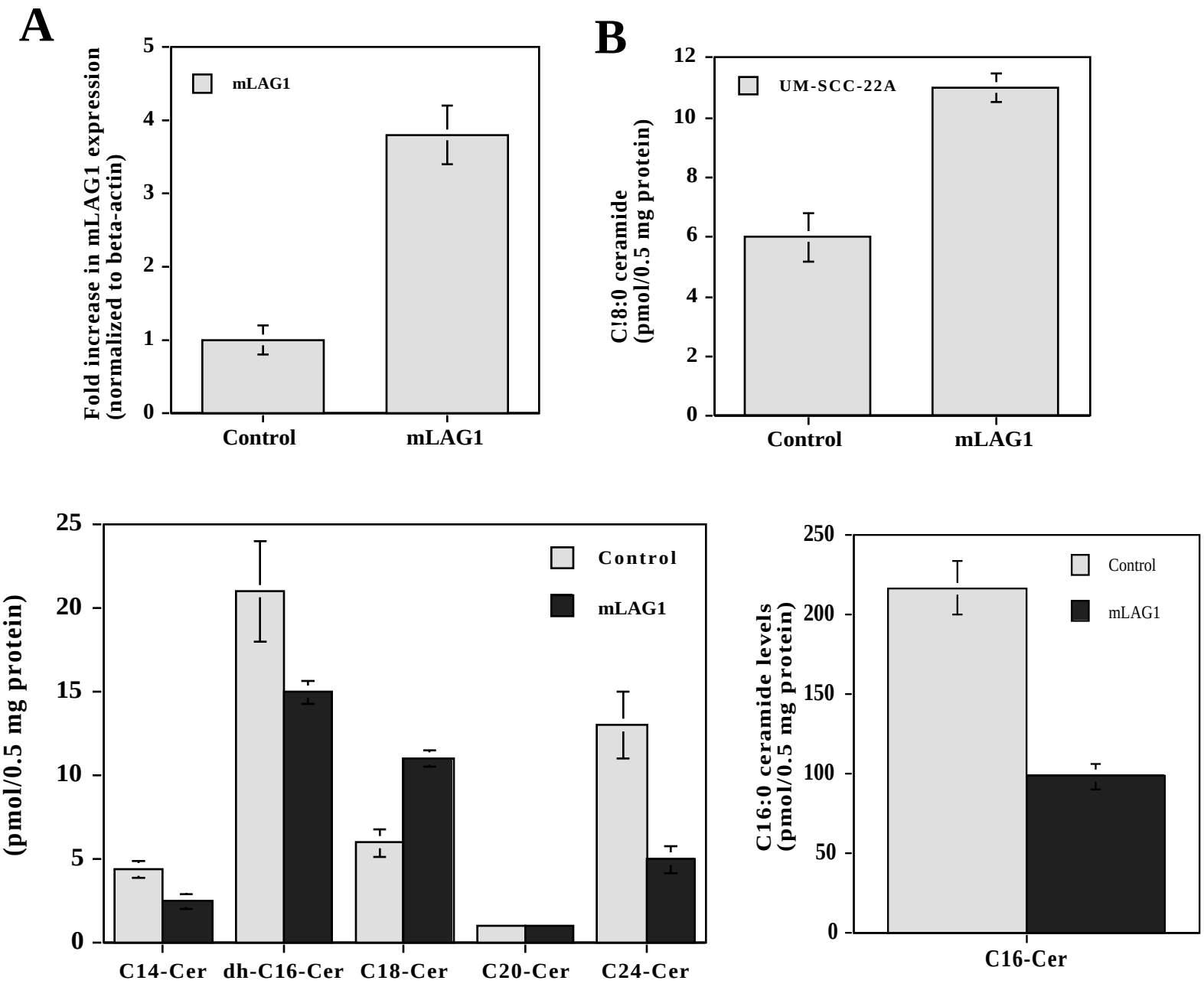

A
Fold increase in mLAG1 expression (normalized to beta-actin)
mLAG1
Control
mLAG1
B
C!8:0 ceramide (pmol/0.5 mg protein)
UM-SCC-22A
Control
mLAG1
C
Cearmide levels (pmol/0.5 mg protein)
Control
mLAG1
C14-Cer
dh-C16-Cer
C18-Cer
C20-Cer
C24-Cer
C16:0 ceramide levels (pmol/0.5 mg protein)
Control
mLAG1
C16-Cer

Figure 5

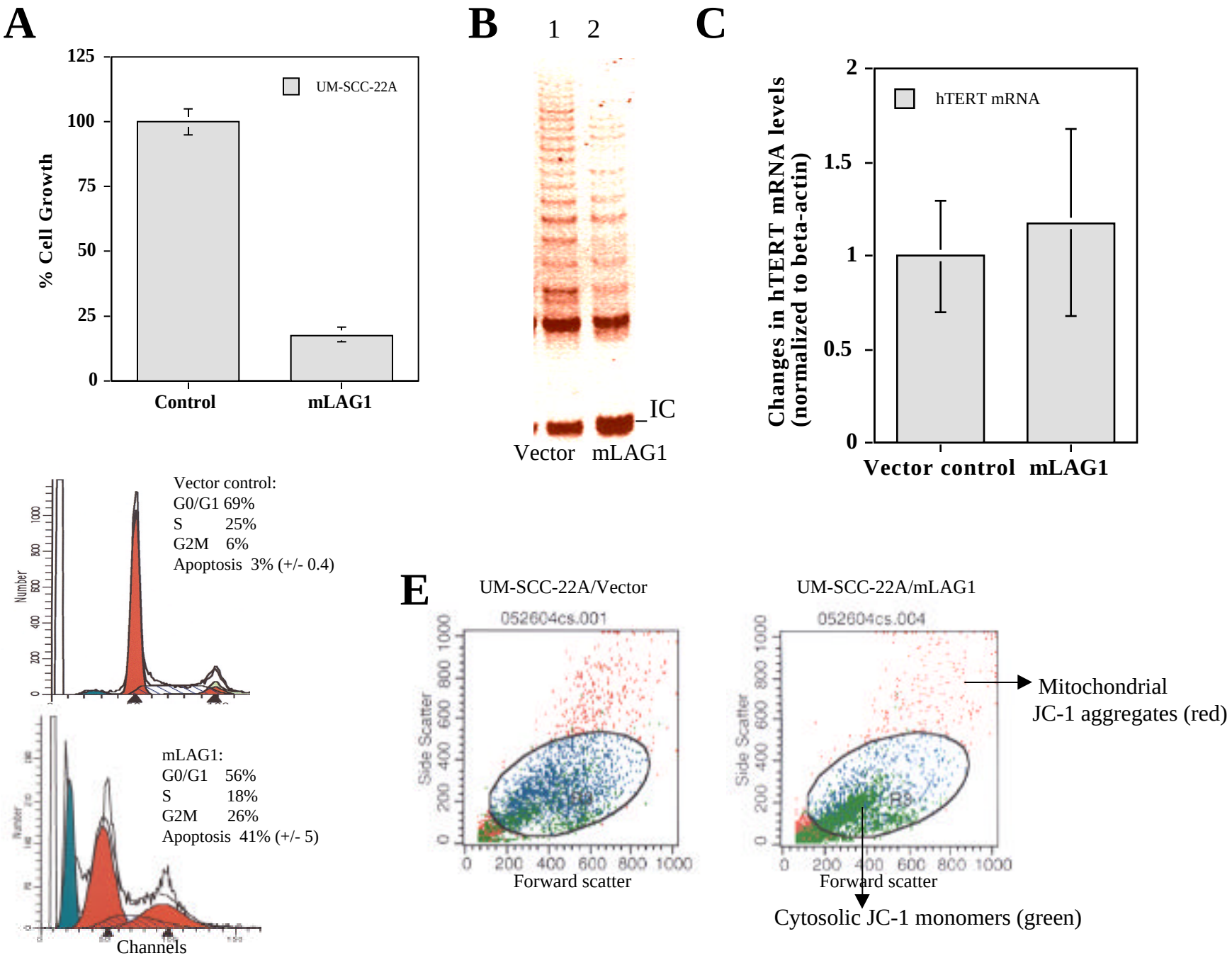
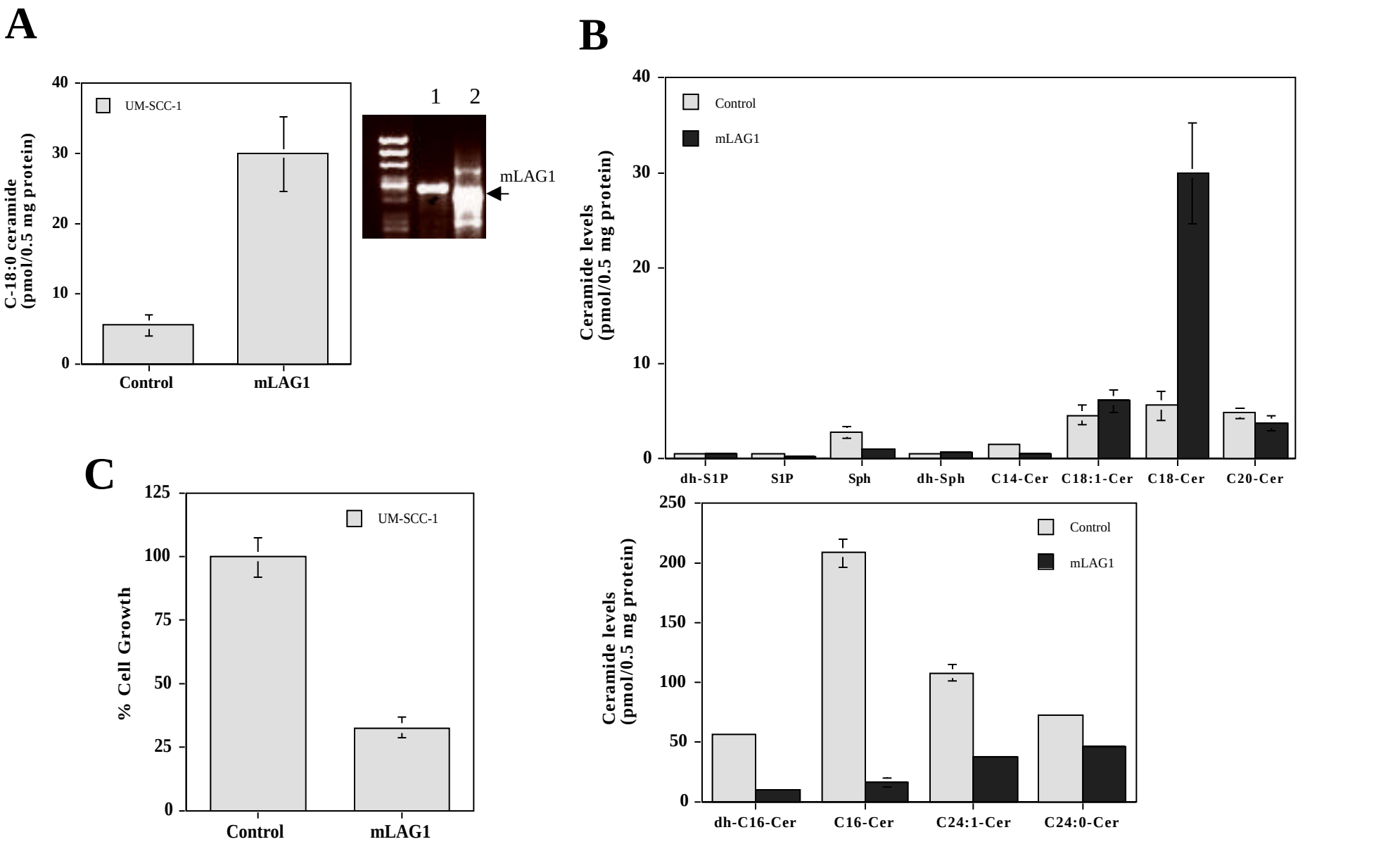

A
125
100
75
50
25
0
% Cell Growth
UM-SCC-22A
Control
mLAG1
B
1 2
_IC
Vector mLAG1
C
2
1.5
1
0.5
0
Changes in hTERT mRNA levels (normalized to beta-actin)
hTERT mRNA
Vector control mLAG1
D
Vector control:
G0/G1 69%
S 25%
G2M 6%
Apoptosis 3% (+/- 0.4)
mLAG1:
G0/G1 56%
S 18%
G2M 26%
Apoptosis 41% (+/- 5)
Number
Channels
E
UM-SCC-22A/Vector
UM-SCC-22A/mLAG1
052604cs.001
052604cs.004
Side Scatter
Forward scatter
R8
Mitochondrial
JC-1 aggregates (red)
Cytosolic JC-1 monomers (green)

Figure 6


A
40
30
20
10
0
C-18:0 ceramide (pmol/0.5 mg protein)
UM-SCC-1
Control
mLAG1
1
2
mLAG1
B
40
30
20
10
0
Ceramide levels (pmol/0.5 mg protein)
Control
mLAG1
dh-S1P
S1P
Sph
dh-Sph
C14-Cer
C18:1-Cer
C18-Cer
C20-Cer
C
125
100
75
50
25
0
% Cell Growth
UM-SCC-1
Control
mLAG1
250
200
150
100
50
0
Ceramide levels (pmol/0.5 mg protein)
Control
mLAG1
dh-C16-Cer
C16-Cer
C24:1-Cer
C24:0-Cer