AD_____

Award Number: DAMD17-02-1-0215

TITLE: Molecular Characterization of Squamous Cell Carcinomas Derived from Recessive Dystrophic Epidermolysis Bullosa

PRINCIPAL INVESTIGATOR: My G. Mahoney, Ph.D.
Ulrich Rodeck, Ph.D.
Jouni Uitto, M.D., Ph.D.

CONTRACTING ORGANIZATION: Thomas Jefferson University
Philadelphia, Pennsylvania 19107

REPORT DATE: June 2003

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	June 2003	Annual (29 May 2002 - 28 May 2003)

4. TITLE AND SUBTITLE
Molecular Characterization of Squamous Cell Carcinomas Derived from Recessive Dystrophic Epidermolysis Bullosa

5. FUNDING NUMBERS
DAMD17-02-1-0215

6. AUTHOR(S)
My G. Mahoney, Ph.D.
Ulrich Rodeck, Ph.D.
Jouni Uitto, M.D., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Thomas Jefferson University
Philadelphia, Pennsylvania 19107

E-Mail: my.mahoney@mail.tju.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Patients with recessive dystrophic epidermolysis bullosa (RDEB) frequently present with squamous cell carcinomas (SCCs) probably as a result of chronic blistering and extensive scarring. These tumors are clinically aggressive as they metastasize readily. The metastasis-associated protein (MTA)-1, a transcription suppressor, is overexpressed in several epithelial neoplasms including SCCs. Our preliminary results demonstrate that MTA1 expression is induced by activation of the epidermal growth factor receptor (EGFR). As deregulation of EGFR signaling is frequently observed in aggressive epithelial neoplasms we propose to study the role of EGFR signaling and MTA1 expression in SCCs derived in RDEB patients. Our Specific Aims are to establish cell lines derived from SCCs in non-RDEB and RDEB patients, characterize the malignant phenotype of these cells as it relates to EGFR expression and signaling and to expression of MTA1, examine the contribution of EGFR/MTA1 to proliferation, invasiveness, and cell survival and identify EGFR-dependent signaling pathways contributing to MTA1 expression in these cells. The results from this research will provide invaluable tools for future analysis of the pathobiology of carcinoma cells and will ascertain whether EGFR/MTA1 signaling pathways contributes significantly to the metastasis and invasiveness of SCC derived from RDEB patients.

14. SUBJECT TERMS
Epidermolysis bullosa, epidermal growth factor, metastasis-associated protein, proliferation, differentiation, survival

15. NUMBER OF PAGES
17

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

INTRODUCTION

Squamous cell carcinoma (SCC) is the second most common form of skin cancer commonly associated with chronic ultraviolet light exposure but they can also arise in association with chronic skin wounds. Epidermolysis bullosa (EB) is heterogeneous group of genetic mechanobullous diseases due to a defective basement membrane zone (BMZ), a distinct structure that separates the epidermis from the underlying dermis. As a result of chronic blistering and extensive scarring of skin tissues in the recessive dystrophic form of epidermolysis bullosa (RDEB), patients frequently develop highly invasive SCCs. The metastasis-associated protein (MTA)-1, a nucleosome-remodeling histone-deacetylase complex protein, is overexpressed in several epithelial neoplasms. The overall goal of our funded research through DAMD17-02-1-0215 is to begin an in depth investigation of RDEB-derived SCC and ascertain whether the epidermal growth factor receptor- (EGFR) and/or the metastasis-associated protein 1- (MTA1) mediated signaling pathways contribute significantly to the highly aggressive malignant phenotype these cells. In order to study the molecular characteristics and mechanisms of metastasis in these cancer cells, we proposed to establish cell lines derived from SCC of RDEB patients and to characterize the malignant phenotype (growth, invasiveness, survival potential, and dependence on EGFR/MTA1 activation) of these cells. Since malignant cancer is characterized by its metastatic and invasive properties, identifying and characterizing the genes that are involved in metastasis will provide the means to predict, select, and treat patients for aggressive therapy. Our **Specific Aim 1** is to establish and characterize epithelial keratinocyte SCC cell lines from RDEB and non-RDEB skin biopsies. **Specific Aim 2** is to characterize the malignant phenotype (growth, invasiveness, and survival potential) of these cells as it relates to EGFR expression and signaling and to expression of MTA1. **Specific Aim 3** is to examine the contribution of EGFR/MTA1 to proliferation,

invasiveness, and cell survival. Finally, **Specific Aim 4** is to identify the signaling pathways downstream of EGFR activation that are relevant to MTA1 expression in SCC from RDEB and non-RDEB.

BODY

This progress report will focus on accomplishments covered in Specific Aims 2 and 3. This work was published in a reputable journal attached in appendix.

Specific Aim 1: Establish and characterize keratinocyte cell lines from SCC biopsies obtained from RDEB and non-RDEB patients.

In order to study the biology of tumor cells from RDEB, we proposed to establish these cells in culture. This is a critical part of this proposal because these cells will serve as excellent tools for any future studies involving keratinocyte tumor biology. We are currently awaiting approval from the Office of Regulatory Compliance and Quality for Human Subjects Protection. Upon receiving approval to collect skin samples from RDEB patients, we will initiate this part of the project. In this past budgeted year, we have established keratinocyte cell lines from normal human skin. Furthermore we have obtained through American Tissue-Type Culture (ATTC) eight non-RDEB epithelial cell lines representing different stages of keratinocyte tumor progression (non-tumorigenic HaCaT (skin), tumorigenic Detroit 562 (pharynx), SiHa (uterine cervix), FaDu (pharynx), A431 (skin), SCC9 (tongue), SCC12 (skin), and SCC13 (skin)). We are currently maintaining these cells in culture and will use them to compare to the RDEB-SCCs.

Specific Aim 2: Determine the expression level and cellular localization of MTA1 in keratinocytes from normal human epidermis and from SCC.

Some results from this Specific Aim have been published (please see attached appendix). To assess MTA1 protein expression, we generated a polyclonal antiserum to a peptide unique to MTA1. By immunohistochemical analysis we showed nuclear staining in keratinocytes throughout the nucleated epidermis. In non-RDEB squamous cell carcinoma skin tissues tested, MTA1 staining was dramatically enhanced compared to normal epidermis and appeared to be localized to the cytoplasm as well as nucleus. We also compared MTA1 expression in normal and malignant keratinocytes. Normal keratinocytes (non-invasive) and HaCaT (immortalized non-tumorigenic) cells expressed low to moderate level of MTA1 as compared to highly invasive A431 squamous carcinoma cells. Upon obtaining RDEB-derived SCCs, we will determine the expression level of MTA1 in both tissues and cells. The results obtained here will determine conclusively whether the expression level of MTA1 directly correlates with the invasive potential of SCCs.

Specific Aim 3: Determine the role of MTA1 in Modulating Proliferation, Differentiation, Invasiveness, and Survival of Epithelial Cells.

Some results from this Specific Aim have been published (please see attached appendix). Although we provided evidence that MTA1 is overexpressed in tumor cells, there is no direct evidence that MTA1 is either necessary for or can by itself promote invasion of tumor cells. To study functional aspects of MTA1 expression in epithelial keratinocytes, we established HaCaT cell variants that expressed *MTA1* cDNA in both, the sense or antisense orientation using a tetracycline-regulatable expression system. To assess transgene expression HaCaT-Mock (vector alone), HaCaT-MTA1-S (sense), and HaCaT-MTA1-AS

(antisense) expressing cells were grown in the presence of absence of tetracycline followed by Western blot analysis of MTA-1 expression. Under these conditions, HaCaT-MTA1-S cells expressed markedly higher MTA1 levels than mock-transfected cells whereas HaCaT-MTA1-AS cells expressed barely detectable MTA1 protein. We showed that over- or under-expression of MTA1 did not immediately affect cell proliferation. Using Alamar Blue dye (an indicator of oxidative metabolism), cell counting, and FACS analysis, we determined that MTA1 expression did not affect cellular metabolism, cell numbers, or cell cycle distribution within 3 days after transgene induction. However, prolong suppression of MTA1 expression reduced cell growth and proliferation.

We used the Biocoat® Matrigel® Invasion assay with fetal calf serum as a chemoattractant to assess the metastatic potential of these transfected cells as they migrated from the upper chamber into the lower chamber. MTA1 overexpression in HaCaT-MTA1-S cells was associated with a significant increase in invasive potential as compared to mock-transfected cells. Because HaCaT cells were not normally invasive, abolishing MTA1 expression did not alter their invasive behavior. In addition to increasing cell invasive behavior, we showed that MTA1 expression affected survival of HaCaT cells in the anchorage independent state. We grew the MTA1 transfected cells in forced suspension culture for 24-72 h and examined their ability to reseed on tissue culture-treated plastic. In this setting, expression of MTA1 antisense sequences (reduction of MTA1 expression) markedly reduced the fraction of cells reattaching after 24-72 h of suspension culture. As expected, EGFR blockade by use of the EGFR antagonistic antibody 425 reduced survival of mock-transfected HaCaT cells. Overexpression of MTA1 could not relieve the requirement for EGFR-derived signals for cell survival in the anchorage-independent state. Interestingly overexpression of MTA1 antisense sequences obviated EGF-dependent HaCaT cell survival in forced suspension culture. These results suggest that MTA1 expression is essential to EGFR-dependent HaCaT cell survival in suspension culture.

In years 2-4 of this funding, we will determine whether MTA1 regulates the state of differentiation of these HaCaT-MTA1 cell lines. We will attempt to abolish MTA1 expression in highly invasive cell lines such as A431 and RDEB-SCCs. We hypothesize that if the expression of MTA1 leads to elevated invasiveness, then inhibition of MTA1 expression should suppress/prevent invasiveness. In addition, it would be interesting in the future to determine if HaCaT cells overexpressing MTA1 could develop malignant metastatic tumors when injected into mice.

Specific Aim 4: Determine the EGFR-mediated signal transduction pathways regulating the expression of MTA1.

There is mounting evidence supporting a relationship between aberrant EGFR activation and migratory/invasive properties of epithelial tumor cells. Interestingly epithelial-derived breast and esophageal carcinomas aberrantly express high levels of both EGFR and MTA1. It is our hypothesis that activation of the EGFR and its signal transduction pathways may regulate the expression of MTA1. Our recent published results demonstrated that the EGFR contributes to steady-state expression of MTA1 mRNA and protein (see appendix). We recently began examining the EGFR signal transduction pathways regulating MTA1 expression. We began by examining the effect of MTA1 expression of Bcl-x_L expression. Expression of the anti-apoptotic Bcl-2 family member Bcl-x_L is important for survival of normal epithelial cells including normal keratinocytes and HaCaT cells in forced suspension culture. As MTA1 expression provided a measure of protection to HaCaT cells in suspension, we determined whether inhibiting MTA1

expression affected Bcl-x_L expression. We observed marked downregulation of MTA1 also downregulated of Bcl-x_L protein levels. We conclude that EGFR-dependent MTA-1 expression contributes to Bcl-x_L expression in HaCaT keratinocytes.

During years 2-4 of this funded research, we will further assess the signal transduction pathways from EGFR activation regulating MTA1 expression. In particular we will determine how does EGFR activation regulate MTA1 expression in epithelial cells and whether EGFR-dependent expression of MTA1 contributes to epithelial cell migration.

KEY RESEARCH ACCOMPLISHMENTS

• Establishment of eight non-RDEB epithelial cell lines representing different stages of keratinocyte tumor progression. Cell migration/wounding assay demonstrated that SCC13 and Fadu were highly migratory, A431, SCC9 and SCC12 were moderately migratory, and HaCaT, Siha and Detroit 562 were non-migratory

• Establishment of variants of human immortalized keratinocytes (HaCaT cells) by expressing MTA1 cDNA in both the sense and antisense orientations.

• Forced MTA1 expression enhances migration of immortalized keratinocytes.

• MTA1 expression is necessary but not sufficient for cell survival in the anchorage independent state.

• MTA1 contributes to expression of the anti-apoptotic Bcl-2 family member Bcl-x_L

• MTA1 expression in immortalized keratinocytes depends, in part, on activation of the epidermal growth factor receptor (EGFR).

• In conclusion, EGFR-dependent MTA1 expression supports anchorage-independent epithelial cell survival and migration consistent with a critical role of MTA1 in the metastatic phenotype.

REPORTABLE OUTCOMES

Some of the results mentioned above have been included in a publication (see attached appendix).

CONCLUSIONS

During the first year of this funding we have made tremendous accomplishment in Specific Aims 2 and 3. Some of the results have been recently published in a reputable journal. During year 2 of this funding we plan to complete Specific Aims 2 and 3 while pursuing Specific Aims 1 and 4.

REFERENCES

APPENDICES

One published manuscript attached

Oncogene (2002) 21, 2161–2170

www.nature.com/onc

Metastasis-associated protein (MTA)1 enhances migration, invasion, and anchorage-independent survival of immortalized human keratinocytes

Mỹ G Mahoney*,[1], Anisha Simpson[1], Monika Jost[1], Mariadele Noé[1], Csaba Kari[1], Deanna Pepe[1], Yoo Won Choi[1], Jouni Uitto[1,2] and Ulrich Rodeck[1]

[1]*Department of Dermatology and Cutaneous Biology, Jefferson Medical College, and Jefferson Institute of Molecular Medicine, Thomas Jefferson University, Philadelphia, Pennsylvania, PA 19107, USA;* [2]*Department of Biochemistry and Molecular Pharmacology, Jefferson Medical College, and Jefferson Institute of Molecular Medicine, Thomas Jefferson University, Philadelphia, Pennsylvania, PA 19107, USA*

The human metastasis-associated gene (MTA1), a member of the nucleosome remodeling complex with histone deacetylase activity, is frequently overexpressed in biologically aggressive epithelial neoplasms. Here, we extend this observation to squamous carcinoma cells, which express high levels of MTA1 relative to normal or immortalized keratinocytes. To address functional aspects of MTA1 expression, we established variants of human immortalized keratinocytes (HaCaT cells) by expressing *MTA1* cDNA in both the sense and antisense orientations. We demonstrate that (1) forced MTA1 expression enhances migration and invasion of immortalized keratinocytes; (2) MTA1 expression is necessary but not sufficient for cell survival in the anchorage independent state; (3) MTA1 contributes to expression of the anti-apoptotic Bcl-2 family member Bcl-x_L; (4) MTA1 expression in immortalized keratinocytes depends, in part, on activation of the epidermal growth factor receptor (EGFR). These results establish that, in keratinocytes, MTA1 expression contributes to several aspects of the metastatic phenotype including survival in the anchorage independent state, migration, and invasion. *Oncogene* (2002) **21,** 2161–2170. DOI: 10.1038/sj/onc/1205277

Keywords: anoikis; epidermal growth factor; epithelial cells

Introduction

The metastasis associated 1 (MTA1) protein (Toh *et al.*, 1994, 1995) is representative of a protein family highly conserved through evolution, which also includes the metastasis associated 1-like protein (MTA1-L1) (Futamura *et al.*, 1999), MTA2 (Zhang *et al.*, 1999) and Mta3 (Simpson *et al.*, 2001). In addition, in pancreatic acinar cells, a derivative of the rat *mta1* gene, *ZG29*, has been identified (Kleene *et al.*, 1999). MTA1 contains regions of homology with several immediate early genes (Herman *et al.*, 1999; Paterno *et al.*, 1997) encoding transcription factors involved in cell growth regulation. Recent studies demonstrate that MTA1 and MTA2 are members of the nucleosome-modeling complex with histone deacetylase activity consistent with a role of these proteins in transcriptional regulation (Toh *et al.*, 2000; Zhang *et al.*, 1999). MTA1 was originally identified based on its overexpression in metastatic rat breast cancer (Toh *et al.*, 1994, 1995). However, little is known about functional aspects of MTA1 expression as they relate to either the normal or the transformed cellular phenotype.

Recently, it was shown that, in malignant breast epithelial cells, MTA1 expression is induced by activation of the heregulin/HER2 pathway (Mazumdar *et al.*, 2001). The HER2/c-*erb*B2 receptor is an orphan receptor tyrosine kinase. When expressed at physiological levels it requires heterodimerization with and transphosphorylation by other members of the epidermal growth factor receptor (EGFR) family for activation (Pinkas-Kramarski *et al.*, 1998). Deregulated EGFR activation frequently occurs in epithelial malignancies, including squamous and breast carcinomas (Barnard *et al.*, 1994; Le Jeune *et al.*, 1993; Mukaida *et al.*, 1991; Ozawa *et al.*, 1989). Collectively, these earlier studies raised the question whether EGFR activation may contribute to MTA1 expression in epithelial cells, and, thus, may enhance their malignant potential.

In the present study we focused on functional aspects of MTA1 expression as they relate to the malignant phenotype of transformed human keratinocytes. We describe that MTA1 expression supported several cellular functions relevant to the metastatic phenotype including survival in the anchorage-independent state, migration, and invasion. In addition, we demonstrate that MTA1 expression in immortalized keratinocytes was induced by EGFR activation and was necessary for, but not sufficient to EGFR-dependent, matrix-independent survival of these cells.

*Correspondence: MG Mahoney, Department of Dermatology and Cutaneous Biology, 233 S. 10th St., Room 419 BLSB, Jefferson Medical College, Philadelphia, PA 19107, USA;
E-mail: My.Mahoney@mail.tju.edu
Received 14 September 2001; revised 14 December 2001; accepted 19 December 2001

These results highlight a previously unrecognized role of MTA1 in support of EGFR-dependent survival of epithelial cells in the anchorage-independent state.

Results

Mta1 expression in normal and immortalized epidermal keratinocytes

MTA1 was originally identified in transformed rat mammary epithelial cells (Toh *et al.*, 1994). Subsequent studies demonstrated human *MTA1* mRNA expression also in esophageal squamous cell carcinomas (Toh *et al.*, 1999). These results led us to investigate MTA1 expression patterns in a panel of normal, immortalized, and malignant keratinocyte lines. Immortalized HaCaT keratinocytes were included because they are very similar to normal keratinocytes (Boukamp *et al.*, 1988) but are amenable to stable transfection providing us with the opportunity to manipulate MTA1 expression. To assess MTA1 protein expression, we generated a polyclonal antiserum to a peptide unique to MTA1. Immunohistochemical analysis of adult human epidermis with the anti-MTA1 antiserum (Figure 1a, panel 2) but not the normal rabbit serum (Figure 1a, panel 1) demonstrated nuclear staining of normal keratinocytes *in situ* throughout the nucleated epidermis. In squamous cell carcinoma skin tissues tested, MTA1 staining was dramatically enhanced (representative example of three, Figure 1a, panels 3 and 4) compared to normal epidermis and appeared to be localized to the cytoplasm as well as the nucleus. Immunoblotting analysis of epidermal protein extracts revealed a prominent protein species of expected molecular mass ($\sim$73 kDa) reacting with the MTA1 antiserum (Figure 1b). To compare MTA1 expression in normal and malignant keratinocytes, we assessed steady-state *MTA1* mRNA and protein levels *in vitro*. As determined by RT–PCR, *MTA1* transcripts were present in both cell types, albeit at considerably lower levels in normal keratinocytes relative to a panel of squamous carcinoma cell lines (Figure 2a). *MTA1* expression levels in HaCaT keratinocytes were intermediate, i.e. slightly higher than in primary keratinocytes yet consistently lower than in squamous carcinomas. Low to moderate expression of MTA1 in normal keratinocytes and HaCaT cells as compared to A431 squamous carcinoma cells was confirmed by immunoblotting analysis using the MTA1 antiserum (Figure 2b).

MTA1 is not required for keratinocyte proliferation

To study functional aspects of MTA1 expression in keratinocytes, we established HaCaT cell variants that expressed *MTA1* cDNA in both, the sense or antisense orientation using a tetracycline-regulatable expression system. To assess transgene expression HaCaT-Mock (vector alone), HaCaT-MTA1-S (sense), and HaCaT-MTA1-AS (antisense) expressing cells were grown for 2 days in the presence or absence of tetracycline followed by Western blot analysis of MTA-1 expression. Under these conditions, HaCaT-MTA1-S cells expressed markedly higher MTA1 levels than mock-transfected cells whereas HaCaT-MTA1-AS cells expressed barely detectable MTA1 protein (Figure 3a). Densitometric quantitation of the Western blot analyses of MTA1 expression relative to β-actin signal confirmed the differences in expression levels between the different transfectants (Figure 3b). Immunostaining confirmed the results of the Western blot analysis (Figure 3c). Note that transgene expression was leaky as MTA expression was slightly diminished in MTA1-AS expressing cells and moderately elevated in MTA1-S expressing cells even in the presence of tetracycline. Thus, MTA1 expression in the MTA1-S-transfected cells in the uninduced state was consistently higher than in mock-transfected cells but reproducibly lower than that of induced MTA1-S-transfected cells.

To determine effects of MTA1 expression on cellular metabolism, HaCaT-Mock, HaCaT-MTA-S, and HaCaT-MTA-AS cells were grown for 24, 48 or 72 h in the presence or absence of FCS without tetracycline to induce transgene expression and then treated with Alamar Blue™. As determined by this assay overexpression of either sense or antisense MTA1 sequences had no significant effect on cellular metabolism (data not shown). Furthermore, cellular proliferation was unaffected as determined by counting cells at various time points after transgene induction (data not shown). Similarly, expression of sense and antisense *MTA1* sequences did not detectably affect cell cycle distribution as assessed by FACS analysis of propidium iodide stained cells (data not shown). In summary, MTA1 expression did not affect cellular metabolism, cell numbers, or cell cycle distribution within 3 days after transgene induction.

MTA1 supports keratinocyte migration and invasiveness in vitro

Next, we determined whether MTA1 expression affected keratinocyte migration using an *in vitro* wound-healing assay (Figure 4a). Migration was assessed by scratching a confluent monolayer of cells and monitoring closure of the defect over time. Relative to mock-transfected cells MTA1 overexpressing HaCaT cells demonstrated markedly enhanced migratory behavior whereas MTA1 antisense expressing cells did not migrate at all. Migration was expressed as distance from the wound edge measured 48 h after wounding (Figure 4b). MTA1 overexpression in HaCaT-MTA1-S cells was associated with a significant increase ($P \leqslant 0.05$; Student's *t*-test for unpaired samples) in migratory potential relative to mock-transfected control cells. To assess the contribution of MTA1 to invasion of HaCaT cells, we performed standard Boyden chamber assays using Biocoat® Matrigel® coated membranes and fetal bovine serum as a chemoattractant (Figure 4c).

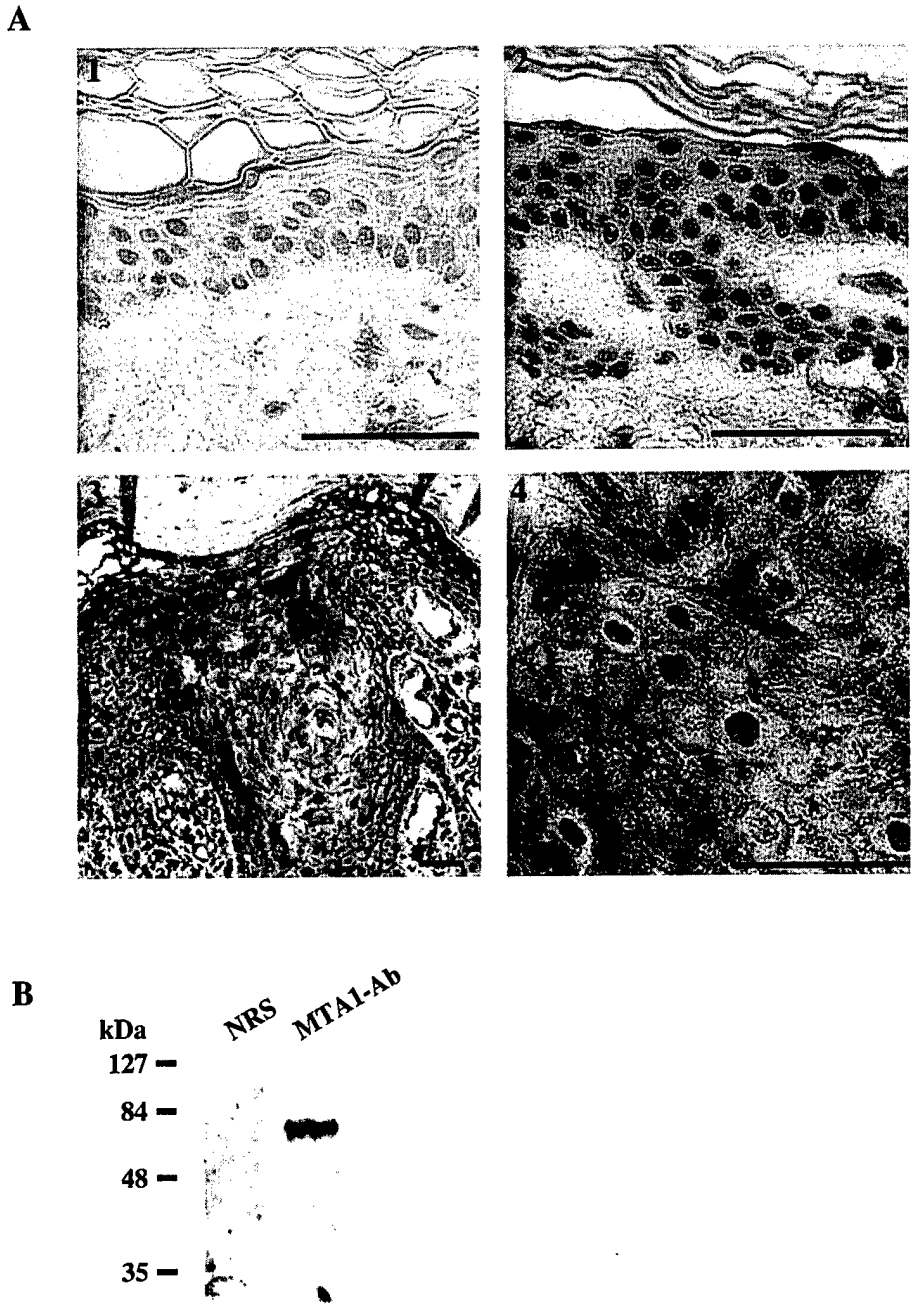

Figure 1 Expression of MTA1 in human epidermis and immortalized keratinocytes. (**a**) Immunohistochemical staining of human epidermis with normal rabbit serum (NRS, panel 1) and anti-MTA1 specific antiserum (panel 2). MTA1 staining was apparent in cell nuclei throughout the epidermis. A representative example of carcinoma malignant skin tissue (SCC) stained for MTA1 (panels 3 and 4). Bar = 50 μm. (**b**) Western blot analysis of immortalized keratinocyte (HaCaT) extracts with the MTA1 antiserum. A protein species of the expected size (approximately 73 kDa) was detected with the anti-MTA1 antiserum but not control NRS

Invasiveness was expressed as the number of cells migrating through Matrigel® relative to migration through uncoated control inserts. MTA1 overexpression in HaCaT-MTA1-S cells was associated with a significant increase ($P \leqslant 0.05$; Student's *t*-test for unpaired samples) in invasive potential as compared to mock-transfected cells. Because HaCaT cells were not normally invasive, abolishing MTA1 expression did not alter their invasive behavior.

MTA1 protects keratinocytes from anoikis

It is believed that invasion and metastatic dissemination of epithelial cells is limited by the inability of normal cells to survive in the absence of appropriate extracellular matrix. In support of this notion, immortalized (Frisch and Francis, 1994; Rodeck *et al*., 1997) and normal (Rodeck *et al*., 1997) cultured keratinocytes undergo apoptosis when denied attachment to extracellular

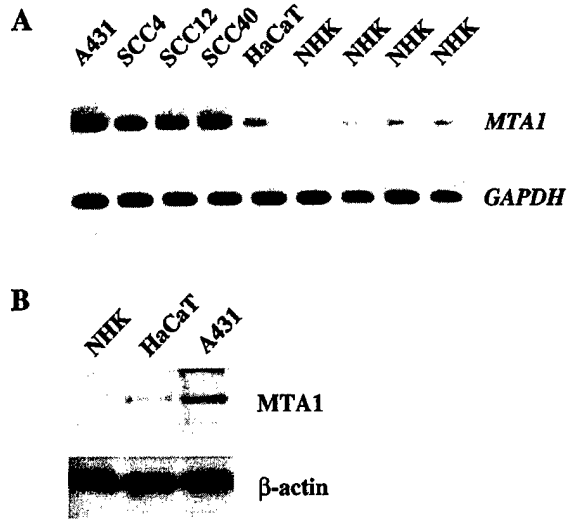

Figure 2 Expression of *MTA1* mRNA and protein in normal keratinocytes and malignant squamous carcinoma cell lines. (a) Semi-quantitative RT–PCR analysis of *MTA1* mRNA expression in four primary human keratinocyte (NHK) strains, immortalized keratinocytes (HaCaT), and several squamous carcinoma cell lines (SCC4,12,40 A431). To account for differences in mRNA concentrations *GAPDH* amplification products were also generated. (b) Expression of MTA1 protein in normal (NHK), immortalized (HaCaT), and malignant (A431) keratinocytes. A431 cells expressed higher levels of MTA1 when compared to NHK or immortalized HaCaT keratinocytes

matrix, in a process termed anoikis. Transformation of epithelial cells with either *Ras* or *Src* oncogenes provides a measure of protection from anoikis induced by forced suspension (Frisch and Francis, 1994; Rosen *et al.*, 2000). Furthermore, EGFR activation by exogenous or endogenous ligands provides partial protection against death to HaCaT cells in suspension (Jost *et al.*, 2001b). Based on these considerations we asked whether MTA1 expression affected survival of HaCaT cells in the anchorage independent state. To this end, we placed HaCaT cells expressing sense and antisense MTA1 in forced suspension culture for 24–72 h followed by transfer to tissue culture-treated plastic and assessment of clonogenic growth 7 days after reseeding. The results of a representative experiment using growth factor-free medium during suspension culture are shown in Figure 5a. In this setting, survival of mock-transfected and MTA1-sense expressing HaCaT cells was comparable. By contrast, expression of MTA antisense sequences markedly reduced the fraction of cells reattaching after 24, 48 and 72 h of suspension culture. As expected, EGFR blockade by use of the EGFR antagonistic antibody 425 (Murthy *et al.*, 1987) reduced survival of mock-transfected HaCaT cells. Importantly, survival of HaCaT-MTA1-S cells was similarly diminished by EGFR blockade suggesting that MTA1 expression alone is not sufficient to relieve the requirement for EGFR-derived signals for cell survival in the anchorage-independent state. Consistent with earlier results (Jost *et al.*, 2001a) addition of EGF to the culture medium during suspension culture markedly improved survival of HaCaT-MTA-S cells (Figure 5b). However, overexpression of MTA1 antisense sequences obviated EGF-dependent HaCaT cell survival in forced suspension culture indicating that MTA1 expression is essential to EGFR-dependent HaCaT cell survival in suspension culture. The concentrations of mAb 425 and EGF have been optimized for this assay as previously published (Jost *et al.*, 2001a). The differences in clonogenic growth after suspension culture were not due to differences in cellular proliferation in the suspended state because forced suspension of HaCaT cells induced cell cycle arrest as assessed by BrdU incorporation (DeHoratius and Rodeck, unpublished results).

Regulation of MTA1 expression through activation of the EGFR

Next, we determined whether expression of MTA1 was regulated through an EGFR-dependent pathway. To this end, we examined the effects of EGFR blockade on expression of MTA1 steady-state mRNA and protein levels in HaCaT cells. Two EGFR antagonists were used in these studies. Whereas the monoclonal EGFR antagonistic antibody 425 (mAb 425) blocks ligand binding and EGFR autophosphorylation, tyrphostin AG1478 (Levitzki and Gazit, 1995) selectively inhibits the tyrosine kinase moiety of the EGFR. Blocking the EGFR with either mAb 425 or AG1478 in attached cells for 2 days was associated with markedly down-regulated *MTA1* mRNA expression in HaCaT cells relative to expression of *GAPDH* (Figure 6a). This result was confirmed by Western blot analysis of MTA1 expression upon inhibition of EGFR signaling (Figure 6b). Treatment of HaCaT cells with either EGFR selective AG1478 or EGFR specific mAb 425 was associated with lower levels of MTA1 expression relative to untreated controls or cells treated with control tyrphostin AG1295 or control mAb BR15-6A. Down-regulation of MTA1 expression was reversible as incubation with either EGF or FCS was able to rescue the effect of mAb 425 treatment (Figure 6c).

MTA1-dependent regulation of Bcl-x_L expression

Previously, we (Rodeck *et al.*, 1997) and others (Frisch and Francis, 1994; Rosen *et al.*, 2000) described that expression of the anti-apoptotic Bcl-2 family member Bcl-x_L is important for survival of normal epithelial cells including normal keratinocytes and HaCaT cells in forced suspension culture. As MTA1 expression provided a measure of protection to HaCaT cells in suspension, we determined whether inhibiting MTA1 expression affected Bcl-x_L expression. We observed marked downregulation of Bcl-x_L protein levels in HaCaT-MTA1-AS cells as compared to HaCaT-MTA1-S cells (Figure 7). As our previous work implicated MEK/MAPK signals in regulation of Bcl-x_L expression levels in HaCaT cells (Jost *et al.*, 2001a), we asked whether inhibition of MEK activity affected MTA1 expression. Neither pharmacological inhibition of MEK activity by PD98059 nor over-

expression of a dominant negative MEK construct (Jost *et al.*, 2001a; Mansour *et al.*, 1994) in HaCaT cells affected MTA1 expression as determined by immunoblot analysis (not shown). This indicates that MEK activity was not involved in regulating MTA1 expression levels in HaCaT keratinocytes. We conclude that EGFR-dependent MTA-1 expression contributes to Bcl-x_L expression in HaCaT keratinocytes.

Discussion

The results presented here highlight a previously unrecognized role of MTA1 in support of epithelial cell survival in the anchorage-independent state. Specifically, we describe that (1) MTA1 is expressed at comparatively low levels in normal and immortalized epidermal keratinocytes; (2) forced MTA1 expression enhances migration and invasion of immortalized keratinocytes; (3) forced MTA1 expression enhances survival of immortalized keratinocytes in forced suspension culture; (4) MTA1 expression in immortalized keratinocytes is controlled, in part, by the EGFR; (5) like the EGFR, MTA1 contributes to expression of the anti-apoptotic Bcl-2 family member Bcl-x_L in keratinocytes and; (6) MTA1 acts in concert with other EGFR targets to achieve cell survival in the anchorage-independent state.

Our results confirm and significantly extend findings published during preparation of this manuscript and relating to MTA1 expression in mammary epithelial cells (Mazumdar *et al.*, 2001). Specifically, forced expression of MTA1 in MCF7 mammary carcinoma cells was associated with enhanced invasion in Boyden chamber assays and increased colony formation in soft agar. Similarly, we observed enhanced migration and invasion of HaCaT keratinocytes overexpressing MTA1. Mazumdar *et al.* (2001) reported no effect of MTA1 overexpression on [^{3}H]-thymidine uptake of MCF7 cells. Similarly, in our hands, neither overexpression nor inhibition of MTA1 expression had any detectable effect on metabolic rates, cell cycle progression, or proliferation of HaCaT keratinocytes. This is in contrast to a previous report describing growth inhibition of human MDA-MB-231 breast cancer cells upon treatment with MTA1 antisense oligonucleotides (Nawa *et al.*, 2000). The reason for this discrepancy is unknown but might relate to differences in cell type expression level of MTA1.

Here, we provide direct evidence that MTA1 expression is required for survival of immortalized keratinocytes in conditions of anchorage independence, i.e. in forced suspension culture. Taken together, the results by Mazumdar *et al.* (2001) and our findings suggest that MTA1-dependent support of epithelial cell survival enhances the capacity of epithelial cells to successfully complete metastatic dissemination to distant sites.

Mazumdar *et al.* (2001) reported upregulation of MTA1 expression in MCF7 breast cancer cells through a heregulin-mediated pathway; heregulin is a ligand for the EGFR-like receptors HER3 and HER4. Our results indicate that EGFR ligands serve a similar role in epithelial keratinocytes. Importantly, both the EGFR and HER3/HER4 activate the orphan receptor HER2. As HER2 is expressed in HaCaT keratinocytes (Ahmed *et al.*, 1997) it is possible that EGFR activation upregulates MTA1 expression through a mechanism involving HER2 transphosphorylation. This possibility is presently under investigation.

Interestingly we observed that HaCaT cells transfected with MTA1-antisense sequences expressed markedly lower levels of Bcl-x_L. We described previously that EGFR-dependent Bcl-x_L expression is required for matrix-independent HaCaT cell survival (Jost *et al.*, 2001b; Rodeck *et al.*, 1997). Furthermore, Bcl-x_L expression in these cells is controlled, in part, by MEK/MAPK signaling (Jost *et al.*, 2001a). Consistent with a role of MTA1 in EGFR-dependent Bcl-x_L regulation we observed that inhibition of EGFR activation was associated with reduced MTA1 expression in HaCaT keratinocytes. However, MTA1 expression in these cells did not depend on MAPK phosphorylation as neither the MEK inhibitor PD98059 nor a dominant negative MEK construct downregulated MTA1 expression. These results suggest that EGFR-dependent MTA1 expression and MEK/MAPK signaling cooperatively support Bcl-x_L expression in keratinocytes. It remains to be investigated whether regulation of Bcl-x_L expression by MTA1 is due to its histone deacetylase activity (Zhang *et al.*, 1999).

A recent study demonstrated that, in melanoma cells selected for survival in suspension culture, resistance to anoikis cosegregates with the ability of these cells to invade across Matrigel barriers (Zhu *et al.*, 2001). It is possible that forced MTA1 expression similarly enhances both, resistance to anoikis and invasive capacity of HaCaT cells. However, we observed that overexpression of MTA1 in HaCaT cells did not provide an unequivocal survival advantage relative to mock-transfected cells when these cells were placed in forced suspension culture. Yet, MTA1 overexpression markedly increased invasion across Matrigel-coated membranes. Thus, it remains to be investigated whether MTA1-dependent increased invasiveness is linked to enhanced survival of HaCaT cells in transit or whether MTA1 enhances the invasive phenotype independently of its role in support of cell survival.

In summary, MTA1 expression appears to be an important component of EGFR-dependent keratinocyte survival in the anchorage-independent state.

Materials and methods

Chemicals and reagents

Purified mouse EGF was from Collaborative Research (Bedford, MA, USA). Monoclonal anti-EGFR antibody (mAb425) binds specifically to EGFR and blocks the binding of both EGF and TGF-α to EGFR (Murthy *et al.*, 1987, 1990; Rodeck *et al.*, 1987). Control mAb BR15-6A (363-15-6A) binds to but does not inhibit the EGFR expressed on epithelial cells (Basu *et al.*, 1987). Tyrphostin AG1478 (Levitzki and Gazit, 1995;

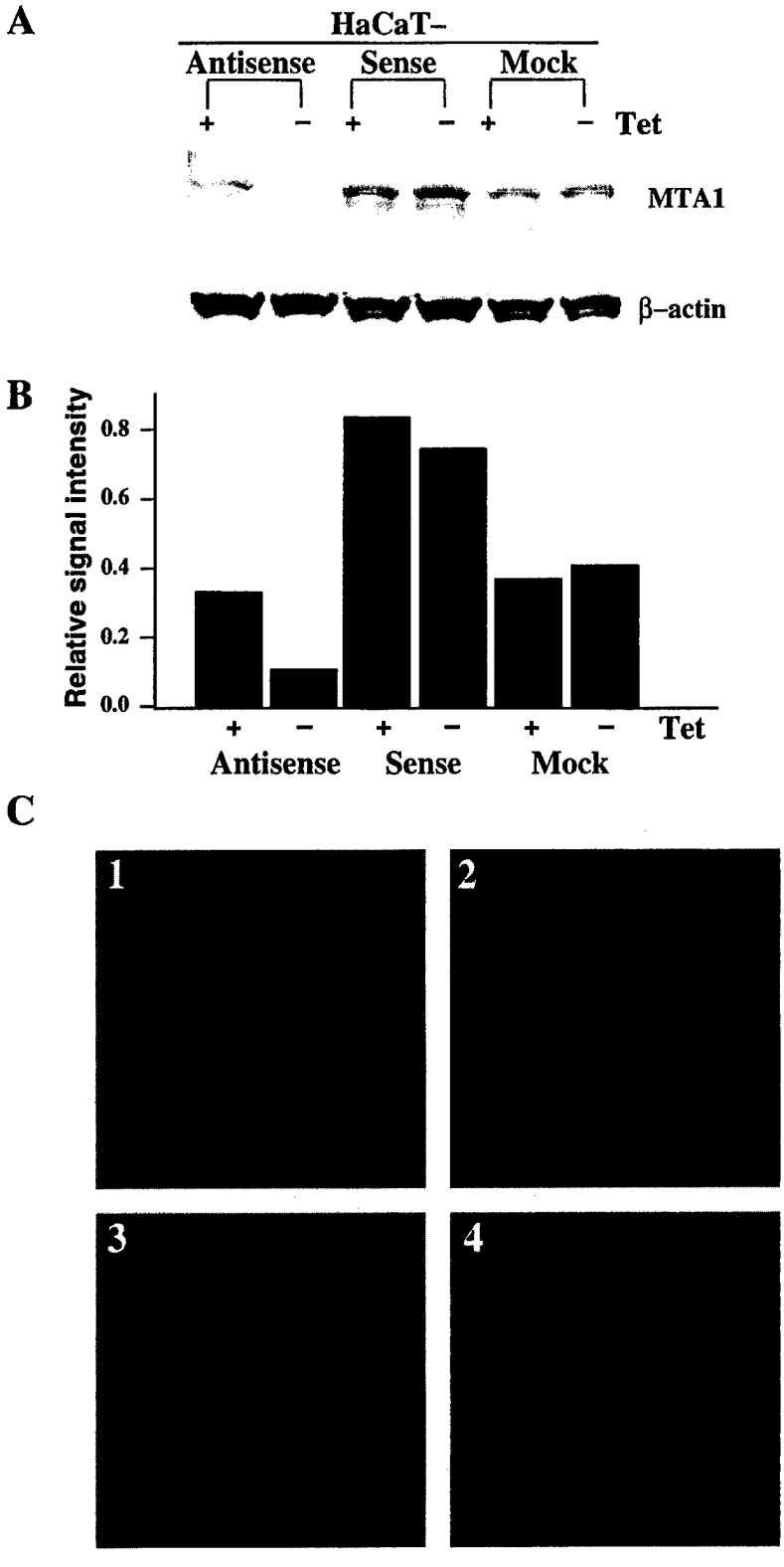

Figure 3 Establishment of HaCaT cells with regulated expression of MTA1 by transfection of sense and antisense *MTA1* sequences using an tetracycline-regulated expression system. (**a**) Western blot analysis of MTA1 expression. Upon transgene expression in the absence of tetracycline, HaCaT-MTA1-S (sense) transfected cells expressed higher MTA1 levels, whereas HaCaT-MTA1-AS (antisense) cells expressed low to undetectable MTA1 levels. Tetracycline removal had no effect on MTA1 expression of HaCaT-Mock cells. (**b**) Densitometric analysis of MTA1 relative to β-actin signal from the Western blotting results above. (**c**) Immunohistochemical detection of MTA1 in mock-transfected HaCaT cells (panel 2) compared to staining with the normal rabbit serum (panel 1). In the absence of tetracycline, MTA1 was overexpressed in the nuclei of HaCaT-MTA1-S cells (panel 3) and barely detectable in HaCaT-MTA1-AS cells (panel 4)

Yoneda *et al*., 1991) and control AG1295 were purchased from Calbiochem (San Diego, CA, USA). DAPI and antibodies to β-actin were from Sigma (St. Louis, MO, USA).

Total RNA preparation, reverse transcription, and semi-quantitative PCR

Total cellular RNA was extracted from cultured cells using the Qiagen RNeasy kit (Qiagen, Valencia, CA, USA) or Tri Reagent (Sigma) according to the manufacturer's protocols. RT–PCR analyses were performed using the Titan kit (Roche Molecular Biochemical, Indianapolis, IN, USA) according to the manufacturer's protocol. Alternatively, cDNA was generated from total RNA using oligo(dT) primers and MMLV reverse transcriptase (Gibco-BRL) in a final volume of 20 μl consisting of 1 μg total RNA, 2.5 μM oligo(dT) primers, 10 mM DTT, 0.5 mM dNTP, and 10 units of MMLV reverse transcriptase. Primers specific to human MTA1 were: forward; 5′-AGC TAC GAG CAG CAC AAC GGG GT-3′; and reverse, 5′-CAC GCT TGG TTT CCG AGG AT-3′. The PCR conditions were 94°C (3 min) and 20–30 cycles of 94°C (1 min), 58°C (1 min), and 72°C (2 min). PCR products were electrophoresed on a 2% SeaKem agarose gel (FMC) and semiquantitative evaluation of *MTA1* RNA expression levels was performed relative to expression of the house keeping genes glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) or *β-actin*. The primers for *GAPDH* were: forward, 5′-ACA GTC CAT GCC ATC ACT GCC-3′; and reverse, 5′-GCC TGC TTC ACC ACC TTC TTG-3′. The primers for *β-actin* were: forward, 5′-GTG GGG CGC CCC AGG CAC CA-3′; and reverse, 5′-CTC CTT AAT GTC ACG CAC GAT TTC-3′. The expected PCR products were 290 bp for *MTA1*, 268 bp for *GAPDH* and 540 bp for *β-actin*.

Preparation of anti-MTA1 antiserum

Based on the rat (GenBank accession number U02522) and mouse (Simpson *et al*., 2001) cDNA sequences, we designed and synthesized the following peptide 'N'-RLDGERPGPNR-NNMSPH-'C' (Genemed Synthesis, San Francisco, CA, USA). This peptide shares sequence homology with the

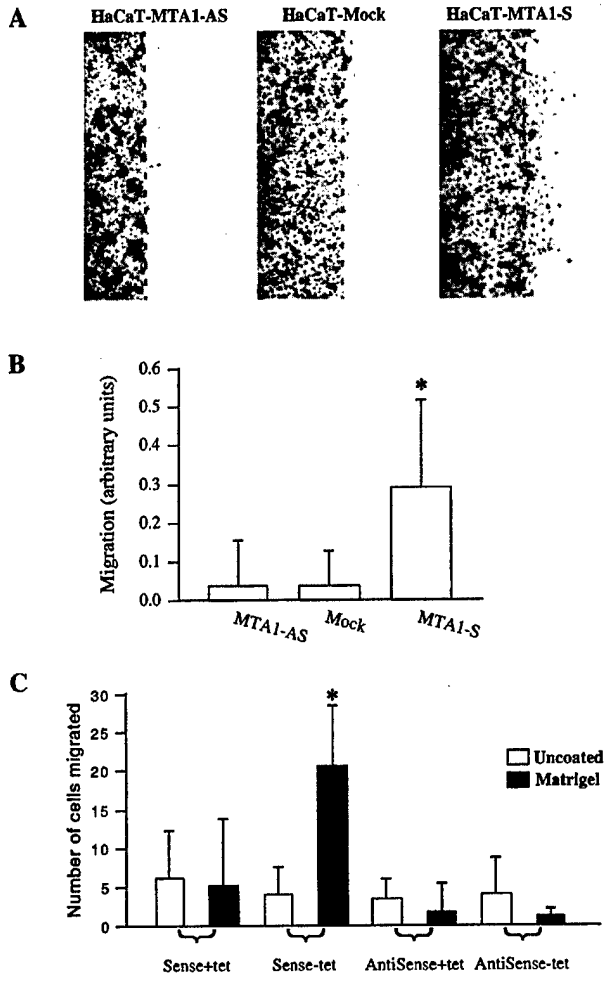

Figure 4 Effects of MTA1 expression on migration and invasion of HaCaT keratinocytes. (**a**) In an *in vitro* cell wounding assay MTA1 sense expressing cells migrated at a faster rate than either mock-transfected or antisense-transfected cells. Migration was evaluated 48 h after wounding by staining cells with crystal violet. Dashed lines demarcate wound edges at the beginning of the experiment. (**b**) Graph shown represents the mean distance of migration ± s.d. of three experiments per condition. (**c**) Boyden chamber assays reveal increased invasiveness in HaCaT-MTA1-S cells when compared to HaCaT-MTA1-AS cells. Invasiveness is expressed as the number of cells that migrated across either uncoated or Matrigel-coated inserts. Results shown represent the mean ± s.d. of 30 fields counted per condition. Asterisks indicate statistically significant differences relative to mock-transfected cells ($P<0.05$; Student's *t*-tests for unpaired samples)

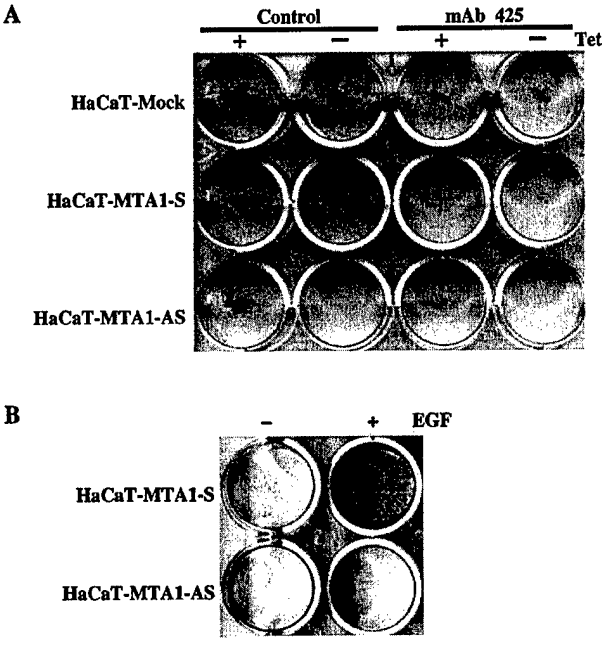

Figure 5 Effects of MTA1 expression on EGFR-dependent survival of keratinocytes in the anchorage-independent state. Clonal growth of cells after 48 h in forced suspension culture was assessed by replating cells on tissue culture-treated plastic and allowing cell proliferation for 7 days after replating. (**a**) HaCaT cells overexpressing MTA1 (HaCaT-MTA1-S) and mock-transfected cells showed comparable survival and regrowth in this setting. By contrast, expression of MTA1 antisense sequences in HaCaT-MTA1-AS cells dramatically reduced the fraction of surviving cells in suspension culture. Addition of the EGFR antagonistic mAb 425 during suspension culture reduced survival of all three cell-lines tested. (**b**) EGFR activation enhanced survival of MTA1-sense expressing HaCaT cells but not of MTA1 antisense-expressing cells

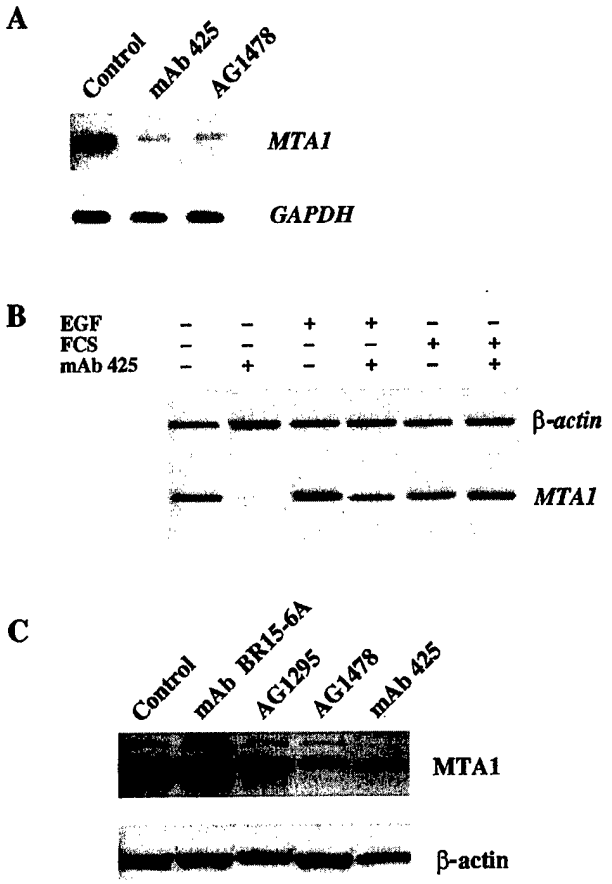

Figure 6 EGFR-dependent MTA1 expression in HaCaT keratinocytes. (**a**) *MTA1* mRNA expression in HaCaT cells as determined by semi-quantitative RT–PCR. HaCaT cells were grown in the absence of exogenous growth factors (Control) or in the presence of mAb 425 or tyrphostin AG1478 as indicated. *GAPDH* expression was determined to account for differences in RNA concentrations. (**b**) Down regulation of MTA1 expression by mAb 425 is reversible. Cells grown in defined medium were treated with FCS (2%) or EGF (50 ng/ml) in the presence or absence of mAb 425 (10 μg/ml). Total RNA was prepared and *MTA1* and *β-actin* levels were determined by RT–PCR. The significant reduction in *MTA1* by mAb 425 was rescued by treatments with either EGF or FCS. (**c**) MTA1 expression in the presence and absence of EGFR antagonists, mAb 425 and AG1478, as determined by Western blot analysis. Reduced levels of MTA1 protein were apparent in extracts of cultures treated with either mAb 425 or AG1478 whereas treatment with control mAb BR15-6A and control tyrphostin had no effect on MTA1 expression. All samples were normalized to β-actin RNA for equal loading

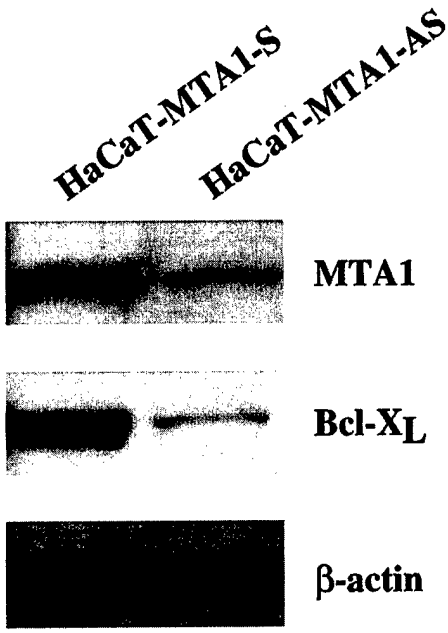

Figure 7 MTA1 expression contributes to Bcl-x_L expression in HaCaT keratinocytes. HaCaT-MTA1-S and HaCaT-MTA1-AS cells were grown for 48 h in the absence of tetracycline. Cell lysates were subjected to Western blot analysis using anti-MTA1 (top), anti-Bcl-x_L (middle), and anti-β-actin (bottom) antibodies. Results of one representative experiment of three are shown

human MTA1 'N'-RLDGERPGPNRSNMSPH-'C' with the exception of the serine residue. This peptide does not share homology with other human MTA proteins, MTA1-L1 or MTA2. The peptide was conjugated to keyhole limpet hemocyanin and used to immunize male New Zealand rabbits and antisera were prepared by CoCalico Biologicals (Reamstown, PA, USA).

Cell lines and tissues

Freshly isolated primary keratinocytes and HaCaT cells were obtained from Dr PJ Jensen (University of Pennsylvania, Philadelphia, PA, USA) and Dr N Fusenig (DKFZ, Heidelberg, Germany), respectively. A431 and SCC lines (SCC4, SCC9, SCC12, and SCC40) were either obtained from the American Type Culture Collection (ATCC, Bethesda, MD, USA) or kindly provided by Dr J Rheinwald (Harvard Institutes of Medicine, Boston, MA, USA). Human neonatal foreskin keratinocyte cultures were initiated and propagated in an MCDB base medium (MCDB153, Sigma) containing 30 μM Ca^{2+} supplemented with ethanolamine, phosphorylethanolamine, hydrocortisone, insulin, purified EGF, and bovine pituitary extract, as previously described in detail (McNeill and Jensen, 1990). HaCaT, A431, and SCC cells were grown in W489 medium consisting of four parts of MCDB153 and one part L15 media supplemented with 2% fetal bovine serum (Rodeck *et al.*, 1991). To inhibit EGFR activation, cells were treated for 2–4 days with mAb 425 (10 μg/ml), control mAb BR15-6A (10 μg/ml), AG1478 (10 μM), or control AG1295 (10 μM) as previously described (Jost *et al.*, 1999).

Expression of MTA1 sense and antisense sequences in HaCaT keratinocytes

Expression of *MTA1* full-length sense and antisense sequences was achieved using an episomally maintained tetracycline (Tc)-regulatable eukaryotic expression vector system based on the *Escherichia coli* Tn10 tetracycline operon (Gossen and Bujard, 1992; Jost *et al.*, 1997). The following primers, forward 5′ -ATG GCC GCC AAC ATG TAC AGG-3′ and reverse 5′-CTA GTC CTC GAT GAC GAT GG-3′, were used to amplify, from human placenta cDNA (Clontech), a 2.2 kb fragment encoding for human MTA1. The PCR conditions were as follows: 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 58°C for 1 min, and 72°C for 2 min. The MTA1 cDNA was

inserted in the sense or antisense orientations into an EBNA-based plasmid vector (pCEPTeTp) that contained the Tc responsive promoter-operator. This construct was transfected into HaCaT cells carrying the plasmid vector tTA encoding the transactivator (HaCaT-tTA, (Jost *et al.*, 1999)). Cells were selected in medium containing neomycin (G418; 400 μg/ml), hygromycin (0.2 μg/ml), and tetracycline (2 μg/ml) for 3 weeks. Expression of MTA1 cDNA in the sense (HaCaT-MTA1-S) or antisense (Ha-MTA1-AS) orientation was induced by removal of tetracycline from the culture medium. Mock-transfected HaCaT cells (HaCaT-Mock) expressed an empty pCEPTetP vector (Jost *et al.*, 1997).

Immunocytochemistry and Western blot analysis

To determine MTA1 expression, cells were washed in ice cold PBS and fixed for 10 min with 50% acetone in methanol at −20°C. After nonspecific sites were blocked for 30 min with 1% BSA in PBS, cells were then incubated with anti-MTA1 (1:100) or anti-α-tubulin (1:1000; Sigma) antibodies for 1 h at room temperature or 4°C overnight. After a 15 min wash with PBS, cells were incubated with Texas-Red-conjugated goat anti-rabbit (1:200) or FITC-conjugated goat anti-mouse (1:200) antibodies for 1 h at room temperature. Samples were washed with PBS and, where indicated, treated with 100 ng/ml DAPI (Sigma) for DNA counterstaining.

To determine MTA1 expression *in situ*, skin sections (5 μm) from formalin-fixed and paraffin-embedded tissues were used. Sections were deparaffinized in 100% xylene (5 min; two times), 100% ethanol (5 min; two times), 95% ethanol (5 min; two times), 75% ethanol (2 min), 50% ethanol (2 min) and H_2O (2 min). Antigen retrieval was performed by incubating sections in 0.3% H_2O_2 for 30 min at room temperature followed by treatment with an antigen retrieval solution (TUF, Signet Labs, Dedham, MA, USA) for 20 min at 95°C. Nonspecific binding sites were blocked with Biotin/Avidin Blocking Kit (Vector Labs, Burlingame, CA, USA) and with BSA (1%). Sections were then incubated with primary antibodies for 2 h at room temperature or overnight at 4°C followed by incubation with biotinylated-goat anti rabbit antibodies (1:200; Vector Labs) for 1 h at room temperature. After washing in PBS for 10 min, sections were incubated in VectaStain ABC (Vector Labs) for 30 min at room temperature, washed in PBS for 10 min and then stained with stable DAB (approximately 1–5 min; Vector Labs). Finally, sections were washed with H_2O, counterstained for 1 min with Gill's #3 Hematoxylin (diluted 1:10 in H_2O; Fisher Scientific, Pittsburgh, PA, USA), washed again with H_2O, mounted in elvanol and examined by light microscopy.

For Western blot analysis, cultured cells were washed in ice cold PBS and scraped into lysis buffer (62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, and protease inhibitor cocktail; Boehringer Mannheim). Cell lysates were vortexed for 30 s, incubated on ice for 30 min, and centrifuged at 12000 *g* for 10 min at 4°C. Supernatants were boiled for 10 min in Laemmli buffer and resolved by 10% SDS–PAGE. Western blot analysis was performed as described previously (Mahoney *et al.*, 1998). Antibodies were used at the following dilutions: rabbit anti-MTA1 antibody (1:200); goat anti-rabbit IgG-horse radish peroxidase (HRP) (1:2000; BioRad Labs, Hercules, CA, USA); monoclonal anti-β-actin antibodies (1:1000; Calbiochem), and goat anti-mouse IgM-HRP (1:1000; Calbiochem).

Cell invasion assay

Biocoat® Matrigel® Invasion chambers (Becton Dickinson Labware, Bedford, MA, USA) were used following a protocol established previously for SCC cells (Kawahara *et al.*, 1995). The assay was carried out using 8 μm tissue culture inserts (Falcon-Fisher Scientific) coated with 90 μg of Matrigel (Becton Dickinson Labware) suspension in PBS per cm^2. Control samples were plated onto uncoated inserts. Briefly, HaCaT-MTA1-S and HaCaT-MTA1-AS cells were grown to 50% confluency in the presence of tetracycline which was then removed from the medium for 2 days to induce transgene expression. The cells were then plated in the upper chambers using serum free medium at 5×10^4 cells/chamber while 10% FBS containing medium was added to the medium in the lower chambers. After incubation for 24 h at 37°C in 95% air/5% CO_2, the non-invading cells in the top chamber were removed. The membranes were fixed with Diff-Quick kit (Dade Behring, Deerfield, IL, USA), and cells which had traversed the side facing the lower chamber were counted using an inverted light microscope (30 optical fields; 2.5 × 2.5 mm grid). Each experimental condition was done in triplicate.

Cell proliferation and viability assays

To assess cell viability and cell proliferation, cells (5×10^4) were grown in 96-well flat-bottom culture dishes in the presence or absence of FCS for 24, 48 or 72 h. Cells were then incubated with 10% Alamar Blue™ (BioSource) in medium for 2 h. The Alamar Blue™ assay provides a measure of the oxidative metabolism of cells. Specifically, the active metabolism of proliferating cells favors a more reduced state as compared to non-proliferating cells. Thus, upon internalization into metabolically active cells the Alamar-Blue™ dye shifts in color from the oxidized indigo blue, non-fluorescing state to the reduced fluorescent pink state. The redox state of Alamar Blue™ was measured spectrophotometrically by monitoring the absorption of Alamar Blue™ at two wavelengths, 530 and 590 nm, in a microtiter plate reader.

In addition, we used FACS analysis to assess cell viability and cell cycle distribution. Cells were trypsinized, washed once with PBS, and fixed with 70% ethanol (−20°C) for 2 h. After a brief wash with PBS, cells were treated with RNAse (1 mg/ml in PBS; Roche Molecular Biochemicals) for 5 min at 37°C and stained with propidium iodide (5 μg/ml in PBS; Molecular Probes) for 20 min at 4°C. Propidium iodide content of cells was analysed with a FACSort cytofluorograph (Becton Dickinson, San José, CA, USA) and Lysis II Software (Becton Dickinson). A minimum of 10^5 cells was accrued for each experimental condition. Furthermore, cellular proliferation was also assessed by determining cell numbers using a hemocytometer.

Forced suspension culture

Forced suspension cultures were performed as described previously (Jost *et al.*, 2001a). Cells were suspended in growth factor-free MCDB base medium containing 0.2% fatty-acid-free bovine serum albumin (Boehringer Mannheim) and seeded at 4×10^5 cells/well in 6-well culture dishes coated with 0.9% agarose gels prepared in the same medium. In select experiments, the base medium was supplemented with EGF (10 ng/ml; Sigma). After 48–72 h, cell aliquots (4×10^5 cells) were reseeded into 12-well culture dishes in W489 medium supplemented with 2% FCS and containing

neomycin, hygromycin, and tetracycline. To assess survival and clonogenic capacity, reattached cells were fixed with 70% ethanol 7 days after reseeding and stained with crystal violet.

Abbreviations

EGFR, epidermal growth factor receptor; MTA1, metastasis-associated protein 1.

Acknowledgments

We thank Dr PJ Jensen (University of Pennsylvania, Philadelphia, PA, USA) for primary keratinocytes, Dr N Fusenig for HaCaT keratinocytes, and Dr J Rheinwald for squamous carcinoma cells. This work was supported by grants from the National Institutes of Health and the Dermatology Foundation to U Rodeck and MG Mahoney, respectively. MG Mahoney is a recipient of a Career Development Award from the Dermatology Foundation.

References

Ahmed NU, Ueda M and Ichihashi M. (1997). *Br. J. Derm.*, **136,** 908–912.

Barnard JA, Graves-Deal R, Pittelkow MR, Du Bois R, Cook P, Ramsey GW, Bishop PR, Damstrup L and Coffey RJ. (1994). *J. Biol. Chem.*, **269,** 22817–22822.

Basu A, Murthy U, Rodeck U, Herlyn M, Mattes L and Das M. (1987). *Cancer Res.*, **47,** 2531–2536.

Boukamp P, Petrussevska RT, Breitkreutz D, Hornung J, Markham A and Fusenig NE. (1988). *J. Cell Biol.*, **106,** 761–771.

Frisch SM and Francis H. (1994). *J. Cell Biol.*, **124,** 619–626.

Futamura M, Nishimori H, Shiratsuchi T, Saji S, Nakamura Y and Tokino T. (1999). *J. Hum. Genet.*, **44,** 52–66.

Gossen M and Bujard H. (1992). *Proc. Natl. Acad. Sci. USA*, **89,** 5547–5551.

Herman MA, Ch'ng Q, Hettenbach SM, Ratliff TM, Kenyon C and Herman RK. (1999). *Development*, **126,** 1055–1064.

Jost M, Class R, Kari C, Jensen PJ and Rodeck U. (1999). *J. Invest. Dermatol.*, **112,** 443–449.

Jost M, Huggett TM, Kari C and Rodeck U. (2001a). *Mol. Biol. Cell*, **12,** 1519–1527.

Jost M, Huggett TM, Kari C, Boise LH and Rodeck U. (2001b). *J. Biol. Chem.*, **276,** 6320–6326.

Jost M, Kari C and Rodeck U. (1997). *Nucleic Acids Res.*, **25,** 3131–3134.

Kawahara E, Imai K, Kumagai S, Yamamoto E and Nakanishi I. (1995). *J. Cancer Res. Clin. Oncol.*, **121,** 133–140.

Kleene R, Zdzieblo J, Wege K and Kern H. (1999). *J. Cell Sci.*, **112,** 2539–2548.

Le Jeune S, Leek R, Horak E, Plowman G, Greenall M and Harris AL. (1993). *Cancer Res.*, **53,** 3597–3602.

Levitzki A and Gazit A. (1995). *Science*, **267,** 1782–1788.

Mahoney MG, Aho S, Uitto J and Stanley JR. (1998). *J. Invest. Dermatol.*, **111,** 308–313.

Mansour SJ, Matten WT, Hermann AS, Candia JM, Rong S, Fukasawa K, Vande Woude GF and Ahn NG. (1994). *Science*, **265,** 966–970.

Mazumdar A, Wang RA, Mishra SK, Adam L, Bagheri-Yarmand R, Mandal M, Vadlamudi RK and Kumar R. (2001). *Nature Cell Biol.*, **3,** 30–37.

McNeill H and Jensen PJ. (1990). *Cell Regul.*, **1,** 843–852.

Mukaida H, Toi M, Hirai T, Yamashita Y and Toge T. (1991). *Cancer*, **68,** 142–148.

Murthy U, Basu A, Rodeck U, Herlyn M, Ross AH and Das M. (1987). *Arch. Biochem. Biophys.*, **252,** 549–560.

Murthy U, Rieman DJ and Rodeck U. (1990). *Biochem. Biophys. Res. Commun.*, **172,** 471–476.

Nawa A, Nishimori K, Lin P, Maki Y, Moue K, Sawada H, Toh Y, Fumitaka K and Nicolson GL. (2000). *J. Cell Biochem.*, **79,** 202–212.

Ozawa S, Ueda M, Ando N, Shimizu N and Ab O. (1989). *Cancer*, **63,** 2169–2173.

Paterno GD, Li Y, Luchman HA, Ryan PJ and Gillespie LL. (1997). *J. Biol. Chem.*, **272,** 25592–25595.

Pinkas-Kramarski R, Lenferink AE, Bacus SS, Lyass L, van de Poll ML, Klapper LN, Tzahar E, Sela M, van Zoelen EJ and Yarden Y. (1998). *Oncogene*, **16,** 1249–1258.

Rodeck U, Herlyn M, Herlyn D, Motlhoff C, Atkinson B, Varello M, Steplewski Z and Koprowski H. (1987). *Cancer Res.*, **47,** 3692–3696.

Rodeck U, Jost M, DuHadaway J, Kari C, Jensen P, Risse B and Ewert D. (1997). *Proc. Natl. Acad. Sci. USA*, **94,** 5067–5072.

Rodeck U, Melber K, Kath R, Menssen HD, Varello M, Atkinson B and Herlyn M. (1991). *J. Invest. Dermatol.*, **97,** 20–26.

Rosen K, Rak J, Leung R, Dean NM, Kerbel RS and Filmus J. (2000). *J. Cell Biol.*, **149,** 447–455.

Simpson S, Uitto J, Rodeck U and Mahoney MG. (2001). *Gene*, **273,** 29–39.

Toh Y, Kuninaka S, Endo K, Oshiro T, Ikeda Y, Nakashima H, Baba H, Kohnoe S, Okamura T, Nicolson GL and Sugimachi K. (2000). *J. Exp. Clin. Cancer Res.*, **19,** 105–111.

Toh Y, Kuwano H, Mori M, Nicolson GL and Sugimachi K. (1999). *Br. J. Cancer*, **79,** 1723–1726.

Toh Y, Pencil SD and Nicolson GL. (1995). *Gene*, **159,** 97–104.

Toh Y, Pencil SD and Nicolson GL. (1994). *J. Biol. Chem.*, **37,** 22958–22963.

Yoneda T, Lyall RM, Alsina MM, Persons PE, Spada AP, Levitzki A, Zilberstein A and Mundy GR. (1991). *Cancer Res.*, **51,** 4430–4435.

Zhang Y, Ng HH, Erdjument-Bromage H, Tempst P, Bird A and Reinberg D. (1999). *Genes Dev.*, **13,** 1924–1935.

Zhu Z, Sanchez-Sweatman O, Huang X, Wiltrout R, Khokha R, Zhao Q and Gorelik E. (2001). *Cancer Res.*, **61,** 1707–1716.