

UNCLASSIFIED

AD NUMBER
ADB259033
NEW LIMITATION CHANGE
TO Approved for public release, distribution unlimited
FROM Distribution authorized to U.S. Gov't. agencies only; Proprietary Info.; Sep 99. Other requests shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott St., Fort Detrick, MD 21702-5012.
AUTHORITY
USAMRMC ltr, 28 Aug 2002

THIS PAGE IS UNCLASSIFIED

AD _____

Award Number: DAMD17-98-1-8065

TITLE: Functional Analysis of a Novel Transcription Factor That
is Amplified and Overexpressed in Breast Cancer

PRINCIPAL INVESTIGATOR: Paul Yaswen, Ph.D.

CONTRACTING ORGANIZATION: University of California at Berkeley
Berkeley, California 94720

REPORT DATE: September 1999

TYPE OF REPORT: Annual

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

DISTRIBUTION STATEMENT: Distribution authorized to U.S. Government
agencies only (proprietary information, Sep 99). Other requests
for this document shall be referred to U.S. Army Medical Research
and Materiel Command, 504 Scott Street, Fort Detrick, Maryland
21702-5012.

The views, opinions and/or findings contained in this report are
those of the author(s) and should not be construed as an official
Department of the Army position, policy or decision unless so
designated by other documentation.

DTIC QUALITY INSPECTED 4

20001027 015

NOTICE

USING GOVERNMENT DRAWINGS, SPECIFICATIONS, OR OTHER DATA INCLUDED IN THIS DOCUMENT FOR ANY PURPOSE OTHER THAN GOVERNMENT PROCUREMENT DOES NOT IN ANY WAY OBLIGATE THE U.S. GOVERNMENT. THE FACT THAT THE GOVERNMENT FORMULATED OR SUPPLIED THE DRAWINGS, SPECIFICATIONS, OR OTHER DATA DOES NOT LICENSE THE HOLDER OR ANY OTHER PERSON OR CORPORATION; OR CONVEY ANY RIGHTS OR PERMISSION TO MANUFACTURE, USE, OR SELL ANY PATENTED INVENTION THAT MAY RELATE TO THEM.

LIMITED RIGHTS LEGEND

Award Number: DAMD17-98-1-8065

Organization: University of California at Berkeley

Those portions of the technical data contained in this report marked as limited rights data shall not, without the written permission of the above contractor, be (a) released or disclosed outside the government, (b) used by the Government for manufacture or, in the case of computer software documentation, for preparing the same or similar computer software, or (c) used by a party other than the Government, except that the Government may release or disclose technical data to persons outside the Government, or permit the use of technical data by such persons, if (i) such release, disclosure, or use is necessary for emergency repair or overhaul or (ii) is a release or disclosure of technical data (other than detailed manufacturing or process data) to, or use of such data by, a foreign government that is in the interest of the Government and is required for evaluational or informational purposes, provided in either case that such release, disclosure or use is made subject to a prohibition that the person to whom the data is released or disclosed may not further use, release or disclose such data, and the contractor or subcontractor or subcontractor asserting the restriction is notified of such release, disclosure or use. This legend, together with the indications of the portions of this data which are subject to such limitations, shall be included on any reproduction hereof which includes any part of the portions subject to such limitations.

THIS TECHNICAL REPORT HAS BEEN REVIEWED AND IS APPROVED FOR PUBLICATION.



10/17/00

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE September 1999	3. REPORT TYPE AND DATES COVERED Annual (1 Sep 98 - 31 Aug 99)	
4. TITLE AND SUBTITLE Functional Analysis of a Novel Transcription Factor That is Amplified and Overexpressed in Breast Cancer			5. FUNDING NUMBERS DAMD17-98-1-8065	
6. AUTHOR(S) Paul Yaswen, Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of California at Berkeley Berkeley, California 94720 E-MAIL: p_yaswen@lbl.gov			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 Distribution authorized to U.S. Government agencies only (proprietary information, Sep 99). Other requests for this document shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, Maryland 21702-5012.				12b. DISTRIBUTION CODE
13. ABSTRACT (Maximum 200 Words) DNA amplification at chromosome position 20q13.2 is common in breast cancer, correlates with poor prognosis, and may reflect the presence of an important oncogene that has not been previously characterized. A candidate oncogene in this region, ZNF217, whose level of expression matches degree of amplification, has been identified through gene mapping and expression studies. To begin to understand how ZNF217 overexpression contributes to breast cancer progression, in vitro studies are being performed to determine how retrovirally transduced ZNF217 alters the phenotype of human mammary epithelial cells (HMEC) from normal tissue. Several biological assays useful in distinguishing normal HMEC from immortally and tumorigenically transformed cells are being used to compare the ZNF217-transduced cells with control cells. To assay the effect of ZNF217 expression on replicative lifespan, parallel cultures were infected with ZNF217 or a control virus, and then were passaged up to, or when indicated, beyond the passage at which control cultures senesced or died. Preliminary data indicates that amplification/overexpression of ZNF217 may be selected for during human breast cancer progression because it effectively immortalizes susceptible HMEC. Overcoming replicative senescence may be necessary for such cells to accumulate the multiple errors necessary for invasion and metastasis.				
14. SUBJECT TERMS Breast Cancer			15. NUMBER OF PAGES 18	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Limited	

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

 Where copyrighted material is quoted, permission has been obtained to use such material.

 Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

 Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

X In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

X For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

N/A In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

N/A In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.


PI - Signature

9/28/99
Date

TABLE OF CONTENTS

STANDARD FORM	2
FOREWARD	3
TABLE OF CONTENTS	4
INTRODUCTION	5
BODY	5-7
KEY RESEARCH ACCOMPLISHMENTS	7
REPORTABLE OUTCOMES	7
CONCLUSIONS	7
REFERENCES	8-9
APPENDICES	10-17

INTRODUCTION

DNA amplification at chromosome position 20q13 is common in breast cancer, correlates with poor prognosis, and may reflect the presence of an important oncogene which has not been previously characterized. A candidate oncogene in this region, ZNF217 (previously designated ZABC1), whose level of expression matches degree of amplification, has been identified through gene mapping and expression studies. In order to begin to understand how ZNF217 overexpression contributes to breast cancer progression, in vitro studies are being performed to determine how retrovirally transduced ZNF217 alters the phenotype of human mammary epithelial cells (HMEC) from normal tissue. Several biological assays useful in distinguishing normal HMEC from immortal and tumorigenically transformed cells are being used to compare the ZNF217-transduced cells with control cells. Alteration of specific phenotypic properties in HMEC overexpressing ZNF217 will provide direct evidence of the gene's oncogenic potential, and provide information about the biochemical pathways affected.

BODY

The coding sequence of ZNF217 was subcloned into a standard, widely-used retroviral vector, LXS_N [1], for efficient uptake and expression in HMEC. High titer amphotropic stocks of ZNF217 and control retrovirus were prepared using a transient packaging system [2] and used to infect recipient HMEC cultures. Northern analysis showed clear evidence of ZNF217 mRNA overexpression (Fig. 1), and immunoblot analysis using a crude antibody preparation showed evidence of ZNF217 protein expression, in the infected cells (Fig. 2). A separate experiment employing a ZNF217-EGFP fusion construct in a plasmid vector showed preferential nuclear localization of fluorescent signal in transfected COS7 cells, confirming the presence of a functional nuclear localization signal in the ZNF217 sequence (Fig. 3). Interestingly, the ZNF217-EGFP fusion protein exhibited a particularly punctate, speckled pattern of fluorescence in some nuclei.

Since a current major interest in our lab is defining the mechanisms that enable normal, finite lifespan HMEC to overcome replicative senescence and acquire the ability to proliferate indefinitely, and since evidence existed that 20q amplification is associated with overcoming replicative senescence, we looked to see whether overexpression of ZNF217 had an effect on replicative lifespan. The successful identification of a gene that contributes to extended or indefinite lifespan depends upon the use of a target cell population where there is a reasonable likelihood that a single change can alter replicative lifespan. We knew from our previous experiments that cells with extended life (EL) compared to untreated controls could be obtained from reduction mammoplasty-derived HMEC cultures exposed to a chemical carcinogen [3], and that very rarely, such EL cultures yield cell lines with indefinite lifespan. Alternatively, when grown in serum-free MCDB 170 medium, untreated reduction mammoplasty derived HMEC cultures give rise to a "self-selected" population capable of long-term growth before reaching replicative senescence [4]. Neither the EL nor the post-selection HMEC express p16, a negative modulator of Rb phosphorylation, because of methylation of the promoter or mutations sustained in the gene itself [5]. Both the EL and the post-selection HMEC can be immortalized in the absence of changes in Rb [6,7] or p53 [3,8]. We chose to begin our transgene experiments with carcinogen-treated, 184Aa EL and post-selection finite lifespan strain 184 HMEC to assay ZNF217 effects on replicative lifespan. In these experiments, cells were infected with ZNF217 or a control virus, and then parallel cultures were passaged up to or when indicated, beyond, the passage at which control cultures senesced or died. Since we found that expression of ZNF217 did not lead to immediate heterogeneity in HMEC morphology or growth rate, the G418-selected cells were pooled in these studies.

Four independent experiments have been performed to date using 184Aa EL cultures. 184Aa cultures infected with ZNF217 retrovirus generally grew slower than parallel cultures infected with control virus during the first 3 to 4 passages after infection. The 184Aa-ZNF217 mass cultures continued to grow, however, after the control mass cultures had clearly senesced around passage 14 (Fig. 4A & Fig. 5A,B). The 184Aa-ZNF217 cultures were marked at this point by slow, heterogeneous growth. Areas of small, mitotic, senescence associated beta-galactosidase (SA- β gal; [9]) negative cells appeared, interspersed with areas of large, flattened, clearly post-mitotic SA- β gal(+) cells (Fig. 5B). Within 3-5 passages, the cultures grew better and became more homogeneous (Fig. 5C). In 3/4 experiments, continuously growing, apparently immortal, cell lines were obtained. These experiments were complicated by the fact that some of the control cells in 2 of the experiments gave rise to clonal outgrowths that grew well immediately after their initial appearance, with morphologies very different from ZNF217 and other control cells. We knew from past experiments that insertional mutagenesis by retroviruses could induce indefinite growth in 184Aa in rare instances [10]. Southern analysis of retroviral integration sites in one case confirmed that the 184Aa cells with indefinite lifespan that grew in control dishes originated from a few (≤ 4) cells at most. In contrast, the 184Aa-ZNF217 cultures with extended growth potential did not arise as clonal

outgrowths and only became apparent when control cultures became senescent. Two 184Aa-ZNF217 cultures have now been taken past passage 43, and continue to show good uniform growth, suggesting that they have attained full immortalization.

One experiment has been performed thus far using normal finite lifespan HMEC strain 184. 184 cultures infected with ZNF217 retrovirus at passage 12 also grew slower during the first few passages after infection than parallel cultures infected with control virus. Control cultures senesced at passage 20. In contrast, the ZNF217-infected cultures continued to grow (Fig 4B), again slowly and heterogeneously at first (Fig.5E), but better and more homogeneously 4-6 passages later (Fig.5F). These cultures are now at passage 33. In contrast to the carcinogen treated EL cultures, we have never observed spontaneous immortalization or immortalization due to insertional mutagenesis in untreated normal finite lifespan HMEC. Two additional normal finite lifespan cultures, 161 and 239, from different individuals, have been infected with ZNF217 and control viruses to assess the reproducibility of the ability of ZNF217 to extend lifespan in normal HMEC. Although the data is still very preliminary, ZNF217 appears to extend the proliferative lifespan of specimens 161 and 239, as well (data not shown).

The finding that overexpression of ZNF217 can cause immortalization of normal HMEC is a major advance. It indicates that amplification/overexpression of ZNF217 may be selected for during human breast cancer progression because overcoming replicative senescence may be necessary for such cells to accumulate the multiple errors necessary for invasion and metastasis. Only one other cellular oncogene, c-myc, has been noted to cause immortalization of HMEC, and in the case of c-myc, the immortalization is inefficient ([11]; our own unpublished results). While the viral oncogene, HPV16-E6, has been shown to efficiently immortalize normal post-selection HMEC, it is likely that this viral oncogene, unlike cellular genes, has been evolutionarily selected to simultaneously compromise several immortalization suppressing functions [12]. Furthermore, viral oncogenes have not been shown to play roles in most human malignancies, including those of breast.

In post-selection HMEC, fibroblasts, and other cell types, replicative senescence has been correlated with shortened telomeres, while overcoming replicative senescence has been correlated with the acquisition of telomerase, an enzymatic activity that can stabilize or lengthen telomeres. The multi-protein ribonucleoprotein telomerase complex can add telomeric sequences de novo, obviating the end-replication problem in linear chromosomes. Cells maintaining telomerase activity display regulation of telomere length, and can retain indefinite proliferative potential. Telomerase activity is detected in the large majority of human cancer tissues and tumor-derived immortal cell lines, which usually maintain short stable telomeres (mean TRF ~3-7 kb) [13]. In contrast, normal finite lifespan human somatic cells do not display telomerase activity, although low levels are seen in some stem cell populations, and transient high levels can be observed in activated T cells [14]. Although telomerase(-) normal human cells express the RNA component (hTR) of the telomerase complex [15,16], the catalytic component (hTERT) is not expressed [15,16]. Transfection of hTERT into some telomerase(-) human cells [17-19], including post-selection HMEC [7], indicates that it is the limiting component of telomerase activity, and sufficient for producing an indefinite lifespan.

Our previous work examining the steps involved in immortal transformation of benzo(a)pyrene-exposed cultured HMEC showed that acquisition of strong telomerase activity and stabilized telomere lengths did not occur simultaneously with the step that transformed the finite lifespan HMEC to a population with indefinite proliferative potential [20]. Because few individual cells in initial passages of the clonally derived immortalized cultures were capable of indefinite growth, but the mass culture was routinely capable of indefinite growth, we have used the term "conditionally" immortal to describe cells at this stage. Conditionally immortal HMEC, like finite lifespan HMEC, are sensitive to TGF β -induced growth inhibition; all normal HMEC ultimately growth arrest in its presence. In contrast, fully immortal HMEC and breast tumor-derived lines can maintain proliferation indefinitely in the presence of TGF β , although often at a slower rate. In our HMEC immortally transformed after carcinogen exposure, acquisition of telomerase activity was closely correlated with subsequent gradual acquisition of TGF β resistance. We have since shown that transduction of normal and conditionally immortal HMEC with hTERT leads to immediate acquisition of telomerase activity and subsequent acquisition of TGF β resistance, indicating that the two traits are somehow linked (unpublished data).

The conditionally immortal population still needed to undergo an additional step, which we have termed "conversion," in order to attain uniform unlimited growth. The conversion of telomerase(-), TGF β growth-sensitive, conditionally immortal HMEC to telomerase(+), TGF β growth-resistant, fully immortal HMEC starts to occur in p53(+) cells when telomere lengths fall below a critical value, and is accompanied by slow heterogeneous

growth and accumulation of the CDK inhibitor, p57Kip2 [21]. The conversion process is characterized by a prolonged period of poor, heterogeneous growth, coincident with high p57 expression and mean terminal restriction fragments (TRF; an indicator of telomere length) < 3 kb, followed by a gradual attainment of uniform good growth \pm TGF β , strong telomerase activity, and absence of p57 expression. In the three p53(+) immortal HMEC lines we have managed to generate by chemical carcinogen treatment and/or insertional mutagenesis, conversion extended over 12-18 months, with uniformly good growing populations present only 20-35 passages after the lines first appeared. The high frequency and incremental nature of conversion in subcloned populations is more consistent with an epigenetic rather than a genetic origin.

Although loss of p53 itself is not necessary for HMEC immortalization, our new data [10] suggests that the total loss of p53 function as part of the immortalization process may produce indefinite lifespan cell lines with significant initial and long-term differences relative to immortal HMEC which retain p53 function. Specifically, we have shown that p53(-/-) HMEC undergo an accelerated conversion to full immortality; telomerase expression is present at early passage levels, mean TRF length does not decline below 3.5 kb, expression of p57 is not detected, and uniform good growth is attained within 10 passages following isolation of these cell lines. Additionally, the p53(-/-) lines demonstrate many initial structural and numerical abnormalities in their karyotypes, as well as genomic instability with continued passage. While total loss of p53 is not necessary for in vitro or in vivo HMEC transformation, it is possible that an activity controlled by the p53 pathway may be compromised in p53(+) immortal HMEC. A more discrete lesion, leaving functional p53 present, would result in a less deranged phenotype than total p53 loss, and could contribute to the generally less aggressive behavior of breast cancers containing wild-type p53.

ZNF217-overexpressing HMEC resemble conditionally immortal HMEC in that they also undergo a heterogeneous growth stage at the time when control cultures are senescing, before acquiring telomerase activity and uniform indefinite growth potential. Preliminary analysis of ZNF217-immortalized 184 and 184Aa cultures indicates that telomerase activity increases incrementally with passage after the point of replicative senescence in control cells is passed (Fig.6). Thus ZNF217 transduction may provide a suitable reproducible system for studying the molecular and biochemical changes that accompany immortalization and conversion of finite lifespan HMEC to a fully immortal phenotype in order to determine whether these changes are amenable to therapeutic intervention.

In the future, we will continue to characterize any additional effects of ZNF217 on phenotypes associated with tumorigenesis. In addition to the stated studies to be performed, we are examining genomic stability, anchorage dependence, and tumorigenicity of the ZNF217 immortalized HMEC. Comparative genomic hybridization (CGH) is being performed after every 5-10 passages to determine whether ZNF217-induced immortalization is associated with any additional genetic lesions or increased genomic instability. Thus far, analysis of one ZNF217-immortalized 184Aa culture shows that there are some chromosomal changes which accompany the immortalization (Fig.7). This suggests that high ZNF217 expression might promote genomic instability in addition to immortalization in susceptible cells.

KEY RESEARCH ACCOMPLISHMENT

- Overexpression of a retrovirally transduced ZNF217 gene in normal finite lifespan and carcinogen treated extended-life HMEC cultures leads reproducibly to immortalization.

REPORTABLE OUTCOMES

None to date.

CONCLUSIONS

Preliminary data indicates that amplification/overexpression of ZNF217 may be selected for during human breast cancer progression because it effectively extends the replicative lifespan of susceptible cells. Overcoming replicative senescence may be necessary for such cells to accumulate the multiple errors necessary for invasion and metastasis. The data also suggests that ZNF217 overexpressing HMEC may undergo a slow, heterogeneous growth stage similar to that exhibited by HMEC made conditionally immortal by chemical carcinogen treatment. Thus ZNF217 transduction may provide a suitable reproducible system for studying the molecular and biochemical changes that accompany immortalization and conversion of finite lifespan HMEC to a fully immortal phenotype in order to determine whether these changes are amenable to therapeutic intervention.

REFERENCES

1. Miller, A. D., Rosman, G. J., Improved retroviral vectors for gene transfer and expression. *Biotechniques*, 7: 980-990, 1989.
2. Finer, M. H., Dull, T. J., Qin, L., Farson, D., Roberts, M. R., *kat*: a high-efficiency retroviral transduction system for primary human T lymphocytes. *Blood*, 83: 43-50, 1994.
3. Stampfer, M. R., Bartley, J. C., Induction of transformation and continuous cell lines from normal human mammary epithelial cells after exposure to benzo(a)pyrene. *Proc. Natl. Acad. Sci. USA*, 82: 2394-2398, 1985.
4. Hammond, S. L., Ham, R. G., Stampfer, M. R., Serum-free growth of human mammary epithelial cells: Rapid clonal growth in defined medium and extended serial passage with pituitary extract. *Proc. Natl. Acad. Sci. USA*, 81: 5435-5439, 1984.
5. Brenner, A. J., Stampfer, M. R., Aldaz, C. M., Increased p16INK4a expression with onset of senescence of human mammary epithelial cells and extended growth capacity with inactivation. *Oncogene*, 17: 199-205, 1998.
6. Wazer, D. E., Liu, X.-L., Chu, Q., Gao, Q., Band, V., Immortalization of distinct human mammary epithelial cell types by human papilloma virus 16 E6 or E7. *Proc. Nat. Acad. Sci. USA*, 92: 3687-3691, 1995.
7. Kiyono, T., Foster, S. A., Koop, J. J., McDougall, J. K., Galloway, D. A., Klingelhutz, A. J., Both Rb/p16INK4a inactivation and telomerase activity are required to immortalize human epithelial cell. *Nature*, 396: 84-88, 1998.
8. Lehman, T., Modali, R., Boukamp, P., Stanek, J., Bennett, W., Welsh, J., Metcalf, R., Stampfer, M., Fusenig, N., Rogan, E., Reddel, R., Harris, C., p53 mutations in human immortalized epithelial cell lines. *Carcinogenesis*, 14: 833-839, 1993.
9. Dimri, G. P., Lee, X., Basile, G., Roskelley, C., Medrano, E. E., Rubelji, I., Pereira-Smith, O. M., Peacocke, M., Campisi, J., A novel biomarker identifies senescent human cells in culture and in aging skin in vivo. *Proc. Natl. Acad. Sci. USA*, 92: 9363-9367, 1995.
10. Stampfer, M. R., Garbe, J., Wigington, D., Nijjar, T., Wong, M., Yaswen, P., Loss of p53 contributes to and accelerates immortalization of cultured human mammary epithelial cells. in preparation.
11. Wang, J., Xie, L. Y., Allan, S., Beach, D., Hannon, G. J., Myc activates telomerase. *Genes & Dev.*, 12: 1769-1774, 1998.
12. Garbe, J., Wong, M., Wigington, D., Yaswen, P., Stampfer, M. R., Viral oncogenes accelerate conversion to immortality of cultured human mammary epithelial cells. *Oncogene*, 18: 2169-2180, 1999.
13. Kim, N. W., Piatyszek, M. A., Prowse, K. R., Harley, C. B., West, M. D., Ho, P. L. C., Coviello, G. M., Wright, W. E., Weinrich, S. L., Shay, J. W., Specific association of human telomerase activity with immortal cells and cancer. *Science*, 266: 2011-2015, 1994.
14. Bodnar, A. G., Kim, N. W., Effros, R. B., Chiu, C.-P., Mechanism of telomerase induction during T cell activation. *Exp. Cell Res.*, 228: 58-64, 1996.
15. Nakamura, T. M., Morin, G. B., Chapman, K. B., Weinrich, S. L., Andrews, W. H., Lingner, J., Harley, C. B., Cech, T. R., Telomerase catalytic subunit homologs from fission yeast and human. *Science*, 277: 955-959, 1997.
16. Meyerson, M., Counter, C. M., Eaton, E. N., Ellisen, L. W., Steiner, P., Caddle, S. D., Ziaugra, L., Beijersbergen, R. L., Davidoff, M. J., Liu, Q., Bacchetti, S., Haber, D. A., Weinberg, R. A., hEST2, the

putative human telomerase catalytic subunit gene, is up-regulated in tumor cells and during immortalization. *Cell*, 90: 785-795, 1997.

17. Bodnar, A. G., Ouellette, M., Frolkis, M., Holt, S. E., Chiu, C.-P., Morin, G. B., Harley, C. B., Shay, J. W., Lichtsteiner, S., Wright, W. E., Extension of life-span by introduction of telomerase into normal human cells. *Science*, 279: 349-352, 1998.

18. Vaziri, H., Benchimol, S., Reconstitution of telomerase activity in normal human cells leads to elongation of telomeres and extended replicative life span. *Current Biology*, 8: 279-282, 1998.

19. Nakayama, J.-I., Tahara, H., Tahara, E., Saito, M., Ito, K., Nakamura, H., Nakanishi, T., Tahara, E., Ide, T., Ishikawa, F., Telomerase activation by hTERT in human normal fibroblasts and hepatocellular carcinomas. *Nature Genetics*, 18: 65-68, 1998.

20. Stampfer, M. R., Bodnar, A., Garbe, J., Wong, M., Pan, A., Villeponteau, B., Yaswen, P., Gradual phenotypic conversion associated with immortalization of cultured human mammary epithelial cells. *Mol. Biol. Cell*, 8: 2391-2405, 1997.

21. Nijjar, T., Wigington, D., Garbe, J. C., Waha, A., Stampfer, M. R., Yaswen, P., p57/KIP2 loss of heterozygosity and expression during immortal conversion of human mammary epithelial cells. *Cancer Res.*, In press: 1999.

APPENDICES

Fig.1. ZNF217 mRNA expression in retrovirally transduced 184 HMEC.

Fig.2. ZNF217 protein expression in retrovirally transduced 184 HMEC.

Fig.3. Nuclear localization of ZNF217-EGFP fusion protein in transfected cells.

Fig.4. Cumulative population doublings by control and ZNF217-transduced HMEC.

Fig.5. Gradual loss of senescence-associated beta-galactosidase expression in ZNF217-transduced HMEC.

Fig.6. Gradual acquisition of telomerase activity in ZNF217-transduced HMEC.

Fig.7. Comparative Genomic Hybridization data for 184Aa-ZNF217 23p.

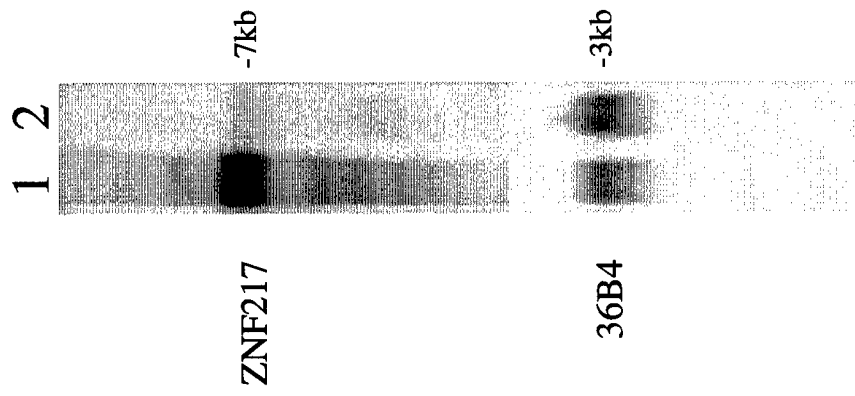


Fig. 1 ZNF217 mRNA expression in retrovirally transduced 184 HMEC.
A Northern blot of total RNA from 184Aa cells infected with a ZNF217-containing retrovirus (lane 1) or infected with the retroviral vector alone (lane 2) was hybridized with a ZNF217 gene fragment. The bottom panel shows the same Northern blot hybridized to a constitutively expressed endogenous gene encoding a ribosomal acidic protein (36B4).

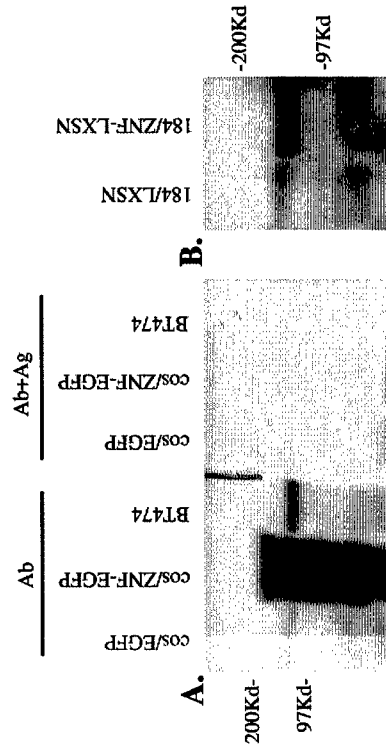


Fig. 2 ZNF217 protein expression in retrovirally transfected 184 HMEC.

A) The specificity of the anti-ZNF217 antibody was demonstrated by duplicate Western blots of whole cell lysates of cos7 cells transfected with a green fluorescent protein (EGFP) construct alone, cos7 cells transfected with a green fluorescent protein-ZNF217 fusion protein (ZNF-EGFP) construct, and endogenous ZNF217-overexpressing human breast tumor cell line BT474. The blot on the left was incubated with an anti-ZNF-217 polyclonal antibody alone (Ab), while the blot on the right was incubated with a combination of the polyclonal antibody and the antigen to which the antibody was produced (Ab+Ag).

B) A Western blot of whole cell lysates of 184 HMEC infected with either the retroviral vector LXSN alone or with the retrovirus containing a ZNF217 gene. The predicted size of unmodified ZNF217 is approximately 125Kd.

EGFP

ZNF-EGFP



Fig. 3. Nuclear localization of ZNF217-EGFP fusion protein in transfected cells.

Cos7 cells were transfected with plasmids containing genes encoding either green fluorescent protein (EGFP) alone or a ZNF217-green fluorescent protein (ZNF-EGFP) fusion protein. Note that fluorescent signal is detected throughout the cell body of a control cell transfected with EGFP alone (left panel), but that fluorescent signal is confined to the nuclei of two cells transfected with the ZNF-EGFP construct (right panel). The fluorescence is particularly bright in speckles found within the nuclei of the ZNF-EGFP transfectants.

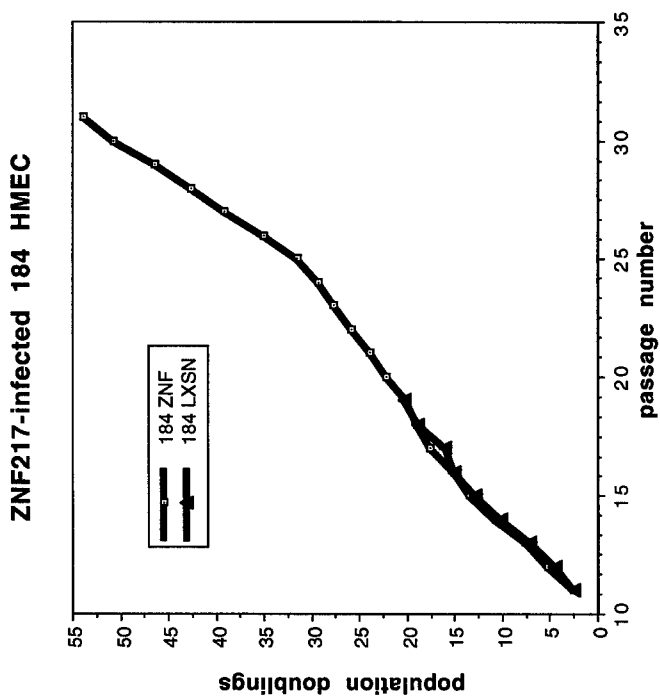
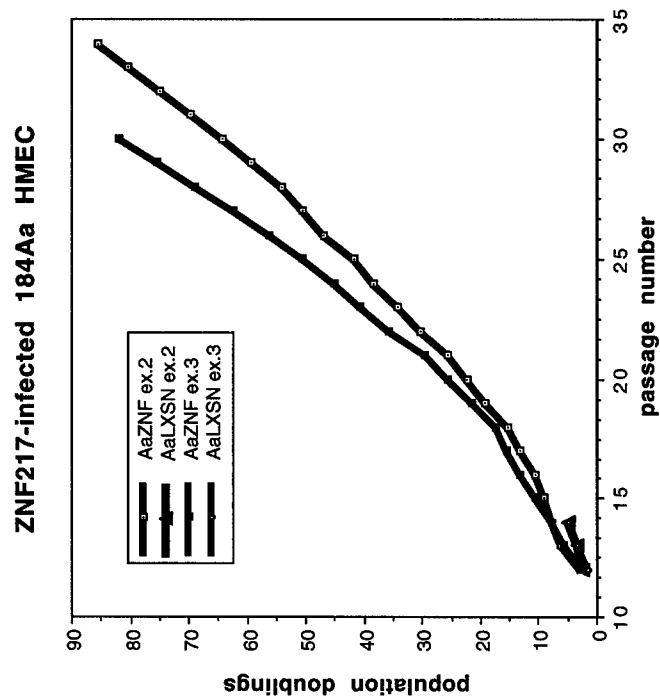


Fig.4 Cumulative population doublings by control and ZNF217-transduced HMEC.
 In the left panel the population doublings of 184 cells infected with either vector alone (LXSN) or ZNF217 plotted against the passage number. The LXSN control senesced at passage number 20.
 In the right panel, the population doubling of 184Aa cells infected with either vector alone (LXSN) or ZNF217 plotted against the passage number for two independent experiments (ex.2 and ex.3). In ex.2 and ex.3, the LXSN controls senesced at passage number 14. In ex.3, an immortal clone grew out of an otherwise senescent population; the growth of this clone is not plotted here.

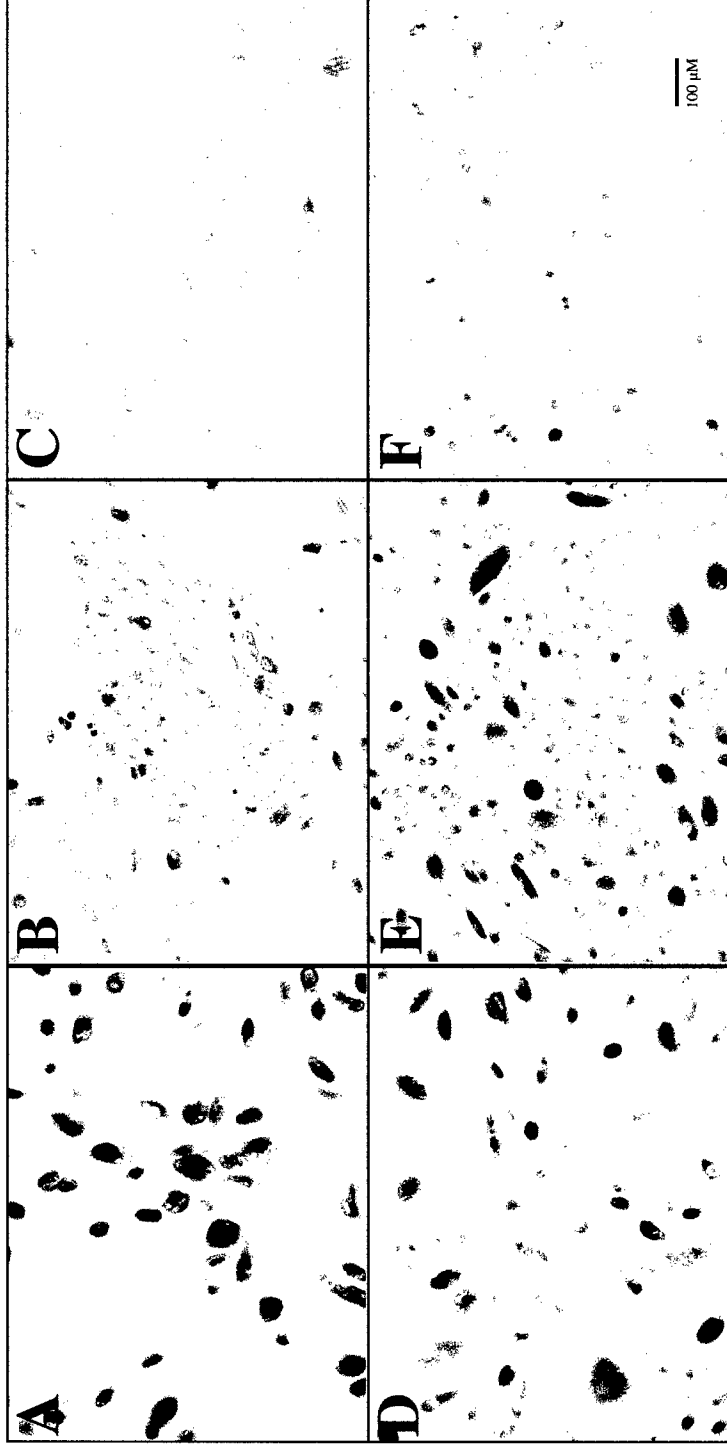


Fig 5. Gradual loss of senescence-associated β -galactosidase expression in ZNF217-transduced HMEC. 184Aa (A-C) and 184 (D-F) HMEC infected with LXS control (A, D) or ZNF217-containing (B, C, E, F) retrovirus were stained for senescence-associated β -galactosidase activity (pH 6.0) at the following passages: (A) p14, (B) p17, (C) p35, (D) p20, (E) p20, (F) p25. Note the presence of small, senescence-associated β -galactosidase negative cells in HMEC infected with ZNF217 virus.

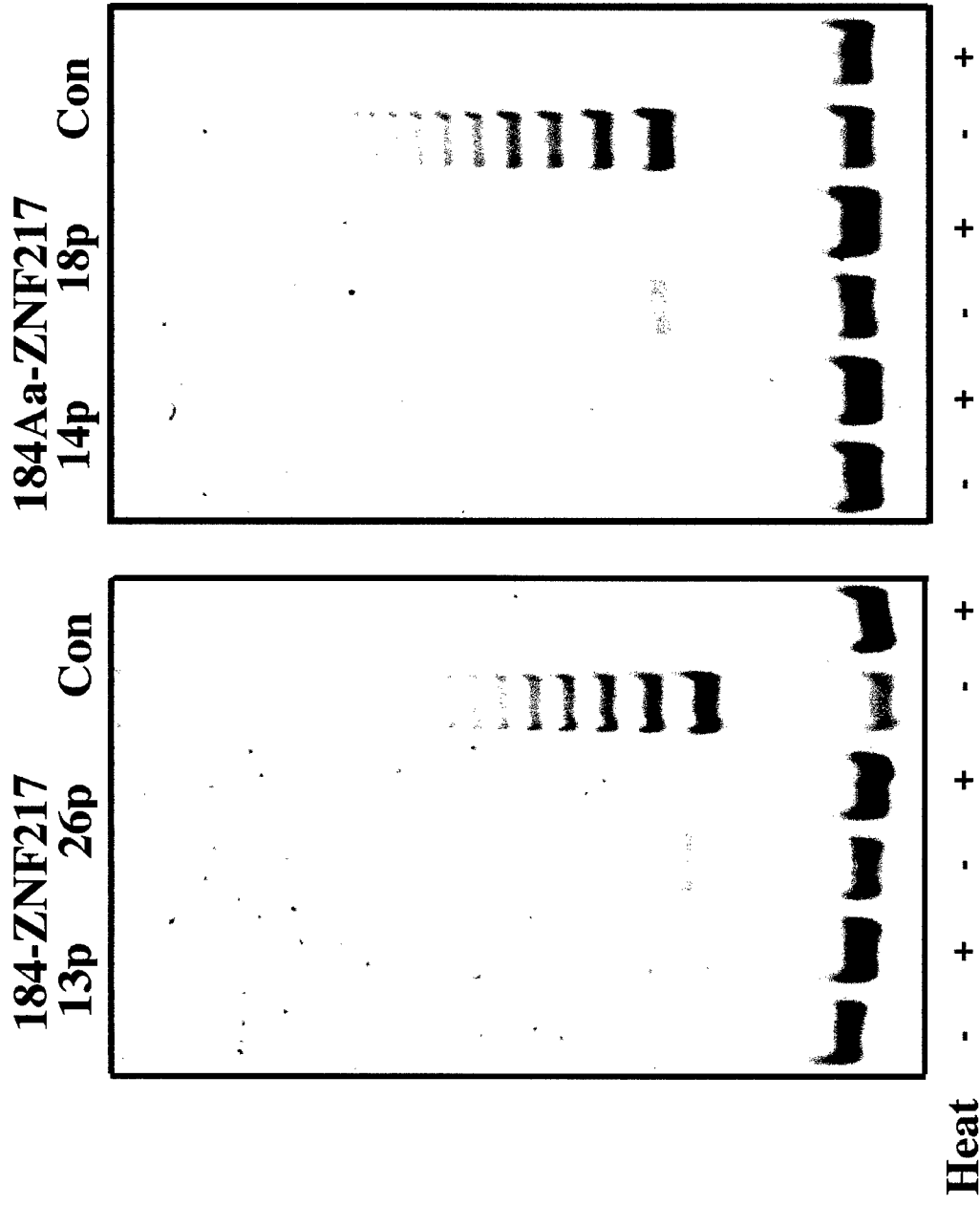


Figure 6. Acquisition of telomerase activity in 184 and 184Aa HMEC infected with ZNF217 retrovirus.

Telomerase activity was measured in 2 μ g extracts of retrovirally transduced cells at the indicated passages and in a positive control immortalized human kidney cell line (Con) using the TRAP-EZE telomerase detection kit. Heat treated extracts were used as negative controls.

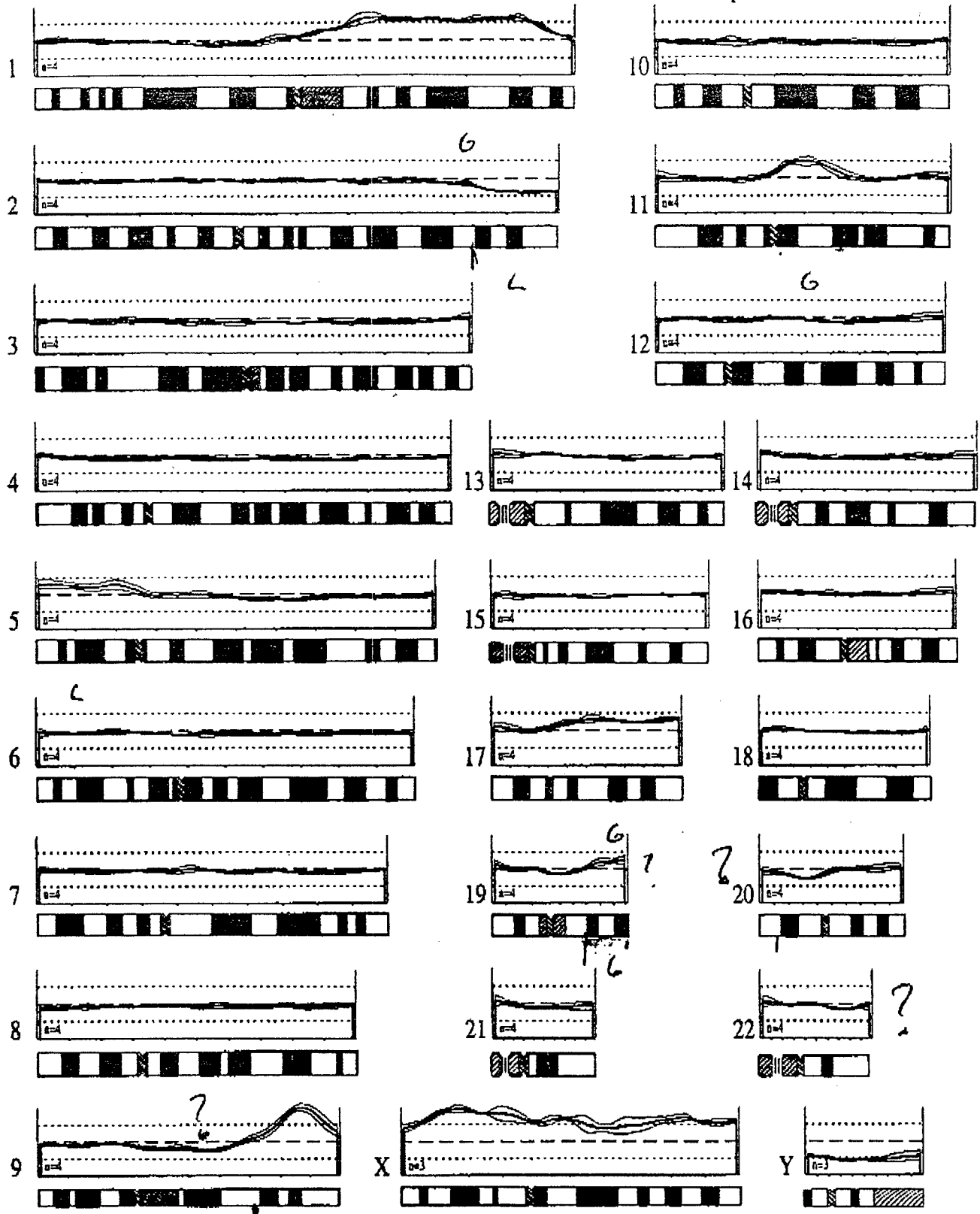


Fig.7. Comparative genomic hybridization reveals DNA-sequence copy-number variation in 184Aa-ZNF217.

Quantitation of green-to-red fluorescence intensities along normal human chromosomes after comparative genomic hybridization with 184Aa-ZNF217 p23 DNA in green and normal DNA in red. The ratios are normalized, so that the average green-to-red ratio for each metaphase cell is 1.00. Note the presence of regional copy-number increases at chromosomes 1q, 5p, 9q, 11q, 17q, 19q(?) and decreases at chromosomes 2q, 9q(?), 20p(?), 22q(?). Question marks denote preliminary data that remain to be verified on additional metaphase spreads.



DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y)

28 Aug 02

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218


SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

Encl


PHYLIS M. RINEHART
Deputy Chief of Staff for
Information Management

ADB231838
ADB240253
ADB251610
ADB275099
ADB253637
ADB261538
ADB275186
ADB264648
ADB275102
ADB241899
ADB259033
ADB266113
ADB275663
ADB254489
ADB262700
ADB276708
ADB274345
ADB274844
ADB275154
ADB275535
ADB275101
ADB275451
ADB274597
ADB273871
ADB275145
ADB274505
ADB275851
ADB274459
ADB27794.2
ADB277404
ADB277494
ADB277536