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13. ABSTRACT ***(Maximum 200 Words)***

The candidate oncogene *ZNF217*, predicted to encode alternately spliced Kruppel-like transcription factors, was originally identified based on its core location in an amplicon on chromosome 20q13.2 in breast cancer cell lines and primary tumors, and its recurrent pattern of expression in tumors. To understand how ZNF217 overexpression contributes to breast cancer progression, in vitro studies are being performed to determine how ZNF217 alters the phenotype of human mammary epithelial cells (HMEC). Initially, we investigated the functional consequences of *ZNF217* overexpression by transducing the gene into finite lifespan HMEC. In five independent experiments, *ZNF217*-transduced cultures maintained growth beyond the point where control cells senesced. HMEC that overcame senescence initially exhibited heterogeneous growth and continued telomere erosion, followed by increasing telomerase activity, stabilization of telomere length, and resistance to TGFβ growth inhibition. This pattern is similar to that observed in rare HMEC lines immortalized following exposure to a chemical carcinogen, where telomerase reactivation and attainment of good uniform growth occurred in an incremental, apparently epigenetic manner, a process we have termed "conversion," as a consequence of overcoming senescence. Aberrant expression of *ZNF217* may be selected for during breast cancer progression because it allows breast cells to overcome senescence and attain immortality.

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INTRODUCTION

The candidate oncogene *ZNF217* (previously designated *ZABC1*), predicted to encode alternately spliced Kruppel-like transcription factors, was originally identified based on its core location in an amplicon on chromosome 20q13.2 in breast cancer cell lines and primary tumors, and its recurrent pattern of expression in tumors (1). 20q amplification, common in many human cancers, is also associated with overcoming senescence and p53-independent genome instability in cultured human uroepithelial cells (2, 3). We investigated the functional consequences of *ZNF217* overexpression by transducing the gene into finite lifespan human mammary epithelial cells (HMEC) (4). In five independent experiments, *ZNF217*-transduced cultures maintained growth beyond the point where control cells senesced. HMEC that overcame senescence initially exhibited heterogeneous growth and continued telomere erosion, followed by increasing telomerase activity, stabilization of telomere length, and resistance to TGFβ growth inhibition. This pattern is similar to what we have observed in rare HMEC lines immortalized following exposure to a chemical carcinogen, where telomerase reactivation and attainment of good uniform growth occurred in an incremental, apparently epigenetic manner, a process we have termed "conversion," as a consequence of overcoming senescence. Aberrant expression of *ZNF217* may be selected for during breast cancer progression because it allows breast cells to overcome senescence and attain immortality.

BODY

Two HMEC strains were used for these experiments: a) 184Aa, an extended but finite lifespan culture obtained from reduction mammoplasty-derived HMEC exposed to a chemical carcinogen (5), and b) 184, a post-selection population of reduction mammoplasty-derived HMEC capable of long-term growth in serum-free medium before reaching senescence (6). The CDK inhibitor $p16^{INK4a}$, thought to serve as one block to immortal transformation (7), is not expressed in either cell strain due to mutation and/or epigenetic silencing (7). Senescence occurs reproducibly after approximately 16 passages (~64 population doublings) in 184Aa and 20 passages (~80 population doublings) in 184 HMEC. In Fig. 1*a*, the cumulative population doublings of transduced cell cultures are plotted against time in culture for two of the three experiments using 184Aa. In both experiments, the *ZNF217*-transduced cells showed no initial growth advantage over the control cultures, but while the latter cultures senesced after 50-100 days, the *ZNF217*-transduced cells continued to grow beyond this point. The control cultures showed large, flat cells with abundant senescence associated β-galactosidase (SA β-gal) activity (8) when they reached senescence (Fig. 2*a*). At similar passages, the *ZNF217*-transduced cultures, termed AaZN1A and AaZN2A, began showing numerous foci of small, mitotic, SA β-gal negative cells among SA β-gal positive senescent cells (Fig. 2*b*). AaZN1A and AaZN2A growth was at first slow and heterogeneous, but became faster and more uniform within 4-6 passages. By later passages, most cells were SA β-gal negative (Fig. 2*c*) and grew well. The third experiment showed similar results.

Since 184Aa cultures have given rise to rare immortal clones spontaneously or by insertional mutagenesis in previous experiments (5) (9) and in these experiments (data not shown), we repeated the *ZNF217* transgene experiments using 184 HMEC, which have never yielded spontaneous immortal clones. The results of the first experiment using 184 were similar to those using 184Aa (Fig. 1*b* & 2*d-f*). Again, the *ZNF217*-transduced cells showed no initial growth advantage over control cells, but continued to grow after the control cells senesced, heterogeneously at first, and faster and more uniformly in later passages, producing the 184ZN4 line (Table 1). A second experiment with 184 HMEC gave a slightly different result - whereas the earlier experiments in 184 and 184Aa showed numerous sites of focal growth at the time of control cell senescence, in this case a single morphologically distinct colony appeared one passage prior to senescence. As in the other experiments, initial passages beyond this point showed very heterogeneous growth.

In post-selection HMEC, as in other cell types, senescence has been correlated with shortened telomeres, and overcoming senescence has been correlated with reactivation of telomerase (10, 11). Cell lines expressing telomerase activity can display regulation of telomere length and retain indefinite proliferative potential. Telomerase activity was not detectable in newly *ZNF217*-transduced 184Aa and 184 cultures, and mean terminal restriction fragment (TRF) size, an indicator of telomere length, continued to decrease in the transduced cultures in the initial passages past the point of control cell senescence (Fig. 3). Telomerase activity then increased incrementally and mean TRF length stabilized at comparatively short lengths, similar to the lengths found in many carcinoma-derived cell lines (12).

All finite lifespan HMEC cease proliferation in response to TGFβ. In contrast, most immortal and malignant epithelial cell lines can maintain growth in its presence, and this trait is thought to contribute to the malignant

phenotype (13). During conversion of carcinogen-induced immortal HMEC, the ability to maintain growth in the presence of TGFβ is acquired incrementally in both mass cultures and clonal isolates (14). The incremental nature and reproducibility of this change suggest that it is due to epigenetic changes in gene expression. Furthermore, the evolution of TGFβ resistance in HMEC transduced with hTERT, the catalytic subunit of telomerase, suggests that the ability to grow in the presence of TGFβ is linked to telomerase reactivation (15). We therefore examined 184ZN4 and AaZN1A at different passage levels for growth capacity in TGFβ. No growth was seen prior to and just after overcoming senescence (Table 1). However, with increasing passage, some cells capable of maintaining growth in the presence of TGFβ began emerging. This gradual, heterogeneous acquisition of TGFβ resistance is similar to what is observed in the carcinogen-immortalized HMEC, although the kinetics are slower in these ZNF217-immortalized lines (14).

Loss of function of the tumor suppressors, p53 and pRb, has been observed in numerous immortal cell lines and is thought to play a role in the immortalization process (16). To determine whether loss of p53 function contributed to the immortalization of the *ZNF217*-transduced HMEC, induction of p53 expression by the DNA damaging agent actinomycin D was measured by immunoblotting. Induction of p53 similar to that in the finite lifespan cells was observed in all three *ZNF217*-transduced immortalized HMEC tested (Fig. 4*a*). For a second confirmation of p53 activity, we analyzed p53-dependent induction of GADD45 transcripts by UV irradiation (17). GADD45 mRNA levels were increased 4 hrs. after UV exposure in both the finite lifespan 184 and immortalized 184ZN4A cultures (Fig. 4*b*). pRb was also present and underwent normal cycles of phosphorylation and dephosphorylation in these cells (data not shown). Thus, as shown previously for the carcinogen-immortalized HMEC (18, 19), alterations in p53 and/or pRb are not obligatory for immortalization of the *ZNF217*-transduced HMEC.

The above data demonstrate that constitutive aberrant expression of *ZNF217* can immortalize finite lifespan HMEC. However, the precise frequency of immortalization has not yet been determined. Southern analysis of retroviral integration sites in *ZNF217*-transduced HMEC growing past senescence suggested that these cultures were rapidly overgrown by distinct clonal populations (data not shown). In an effort to determine whether distinct chromosomal alterations might be conferring growth advantages on clones immortalized with *ZNF217*, DNA from three different immortalized cultures was used for quantitative measurement of DNA copy number using comparative genomic hybridization (CGH) (20). CGH analysis showed low level regional DNA-sequence copy number variations on chromosomes 1q and 8q common to all three cell lines (Fig. 5). The region amplified on 8q included the c-myc oncogene, which itself has been shown to cause HMEC immortalization when overexpressed (21). In addition, each line showed unique regions of high and low level DNA-sequence copy number variations. These sites of regional copy number variation, some of which have also been frequently observed in breast cancer cell lines and primary tumors (20), may contain genes that cooperate with *ZNF217* in facilitating growth and immortalization.

KEY RESEARCH ACCOMPLISHMENTS

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

- Immortalization of *ZNF217*-transduced HMEC occurred without changes in p53 inducibility or function, and without changes in Rb expression.

- Reactivation of telomerase and attainment of uniform good growth +/- TGFβ occurred incrementally after *ZNF217*-transduced HMEC have overcome senescence.

- CGH analysis of three cell lines showed common low level regional DNA-sequence copy number variations on chromosomes 1 and 8 that may be sites of genes that cooperate with ZNF217 in facilitating growth and immortalization.

REPORTABLE OUTCOMES

Manuscript in preparation - Genevieve H. Nonet, Martha R. Stampfer, Koei Chin, Joe W. Gray, Colin C. Collins, and Paul Yaswen The *ZNF217* gene amplified in breast cancers promotes immortalization of human mammary epithelial cells.

Abstract - G. H. Nonet, M.R. Stampfer, C.C. Colins, J.W. Gray, and P. Yaswen. Immortal transformation of human mammary epithelial cells following overexpression of ZNF217: a gene amplified and overexpressed in breast cancer. Proc. Amer. Assoc. Cancer. Res. 41, 318, 2000.

Abstract - P. Yaswen., G. H. Nonet, C.C. Collins, J.W. Gray, and M.R. Stampfer. Immortalization of human mammary epithelial cells by ZNF217: a novel gene amplified and overexpressed in breast cancers. Proc. DOD Breast Cancer Research Program Meeting I, 83, 2000.

Abstract - P. Yaswen, G. H. Nonet, C.C. Collins, J.W. Gray, and M.R. Stampfer. Human mammary epithelial cell immortalization by ZNF217: a novel gene amplified and overexpressed in breast cancers. Telomerase and Telomere Dynamics in Cancer and Aging June 24-28, 2000 San Francisco, CA.

New project funded - "Immortalization of Human Mammary Epithelial Cells by ZNF217" by UC Breast Cancer Research Program, PI - Paul Yaswen.

CONCLUSIONS

The results obtained support the hypothesis that *ZNF217* gene amplification is frequently found in breast cancers because it is involved in enabling breast cells to overcome the restraints of senescence, thus allowing the cells to continue growing and accumulating other changes necessary for malignant progression. The slow gradual changes in telomerase activity and growth in *ZNF217*-transduced cells after they have overcome senescence resemble the changes seen during the conversion process in carcinogen-immortalized HMEC, where measurable telomerase reactivation follows rather than precedes the overcoming of senescence. While viral oncogenes HPV E6 and E7 can also immortalize HMEC (22), HPV is not associated with most human cancers, other than those of the cervix. *ZNF217* transduction, on the other hand, represents a biologically relevant model for one of the changes involved in immortalization and in cancer progression.

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APPENDICES

Fig. 1 *ZNF217*-transduced HMEC continue to grow indefinitely after control cultures have senesced. The cumulative population doublings of **(a)** 184Aa or **(b)** 184 cells infected with either vector alone (LXSN; ●,■,▲), or ZNF217 (○,□,Δ) were plotted against time in days. The LXSN controls senesced and were discarded after greater than 60 days in culture without net increases in cell numbers. In one 184Aa experiment shown, a single immortal clone grew out of an otherwise senescent LXSN-infected population and is not plotted; this clone was distinct from ZNF217-transduced immortal clones in both morphology and growth characteristics. Note that the population doublings indicated are underestimates, since they do not take plating efficiencies into account.

Fig. 2 *ZNF217*-transduced cultures show gradual loss of senescence associated β-galactosidase (SA β-gal) activity. 184Aa **(a-c)** and 184 **(d-f)** HMEC infected with LXSN control **(a,d)** or ZNF217-containing **(b,c,e,f)** retrovirus were stained for SA β-gal activity (pH 6.0) at the following passages: **(a)** p14, **(b)** p17, **(c)** p35, **(d)** p20, **(e)** p20, **(f)** p25. Control cultures showed large, flat cells with abundant SA β-gal staining when they reached senescence at passage 14-16 for 184Aa or passage 20 for 184. At this point, ZNF217 cultures began showing the presence of small, mitotic, SA β-gal (-) cells in a background of positive senescent cells. By later passages, most of the cells were SA β-gal (-) and were growing well.

Fig. 3 Finite lifespan 184 HMEC transduced with *ZNF217* show gradual acquisition of telomerase activity and stabilization of telomere lengths. Telomerase activity at indicated passages was measured in 2μg extracts of 184 HMEC transduced with LXSN alone (negative control) or LXSN containing the *Z NF217* gene (184ZN4). This representative telomerase assay gel reveals the characteristic 6-bp ladder indicative of enzymatic activity which is prominent in an immortalized human kidney cell line (+; positive control) and later passage (26p and 27p) 184ZN4 cells. Heat-treated extracts were used as negative controls. Mean TRF size (an indicator of telomere length) was calculated from Southern blots (data not shown) of genomic DNA harvested from cells at the indicated passages.

Fig. 4 p53 expression and function are intact in HMEC immortalized after *ZNF217*-transduction. **(a)** Immunoblot of p53 expression in response to DNA damage by 24 hr. treatment with indicated concentrations of actinomycin D. 184 has wild type p53. 184AA3 is a negative control HMEC line in which one *TP53* allele has been inactivated by insertional mutagenesis and the other allele has been inactivated by unknown means (Stampfer et al., submitted). The cells were assayed at passages 14 (184), 52 (AaZN1A), 49 (AaZN2A), 39 (184ZN4A), and 45 (184AA3). **(b)** The relative abundance of GADD45 mRNA in indicated cell types 4 hrs. after exposure to UV-irradiation (37 joules/cm^2) was measured by Northern analysis, normalized to the levels of a ribosomal protein transcript, and is presented in graphical form as induction relative to that in the same cells at 0 hrs.

Fig. 5 HMEC immortalized after *ZNF217*-transduction show regional chromosomal copy number variations. The ratio of green to red fluorescence intensities along normal human chromosomes was calculated after comparative genomic hybridization (CGH) with ZNF217-transduced cell DNA in green and normal in red. DNA samples from ZNF217-transduced 184 cells were analyzed at passages 21, 26, and 36. DNA samples from two independent ZNF217-transduced 184Aa cell experiments were analyzed at passages 40 and 55 for AaZN1A, and at passage 36 and 54 for AaZN2A.

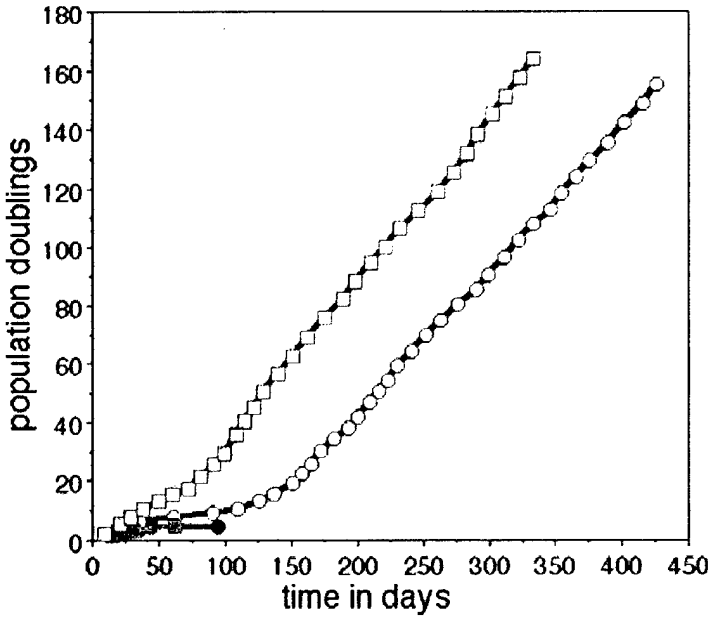
Table 1: Growth of 184ZN4 and AaZN1A colonies at different passage levels in the absence or presence of TGFβ

Passage	Cell	LABELING INDEX (%) TGFβ (-)				TGFβ (+)				CFE(%)
		≤10	10-25	26-50	>50	≤10	10-25	26-50	>50	
184										
13	LXSN	0	3	14	83	100	0	0	0	9.8
	ZNF217	0	9	31	60	95	5	0	0	12.8
17	LXSN	86	14	0	0	100	0	0	0	7.3
	ZNF217	9	16	28	47	100	0	0	0	4.1
20	ZNF217	4	5	35	56	93	7	0	0	4.8
23	ZNF217	0	14	0	86	100	0	0	0	0.75
28	ZNF217	0	0	1	99	100	0	0	0	8.4
43	ZNF217	0	0	0	100	63	21	5	11	11.8
184Aa										
23	ZNF217	0	0	0	100	62	11	11	16	5.5
30	ZNF217	0	0	0	100	71	11	11	7	12.5
44	ZNF217	0	0	0	100	53	8	13	26	9.5
50	ZNF217	0	0	0	100	44	3	2	51	16.8

200-10,000 single cells were seeded per 100 mm dish, and the labeling index ± TGFβ in the ensuing colonies which contained >50 cells was determined as described in Materials and Methods. # colonies refers to the number of colonies counted to determine percentage labeling index.

Fig. 1

a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100
120
140
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180
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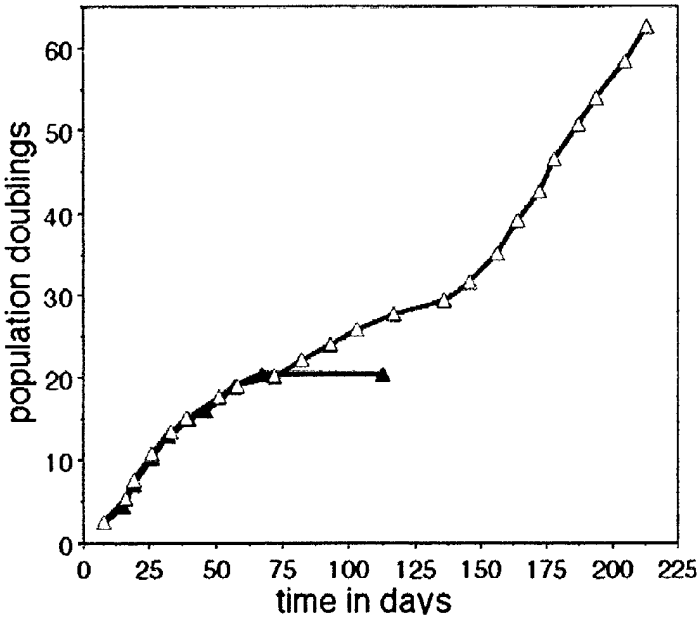
50
100
150
200
250
300
350
400
450

b


population doublings
time in days
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50
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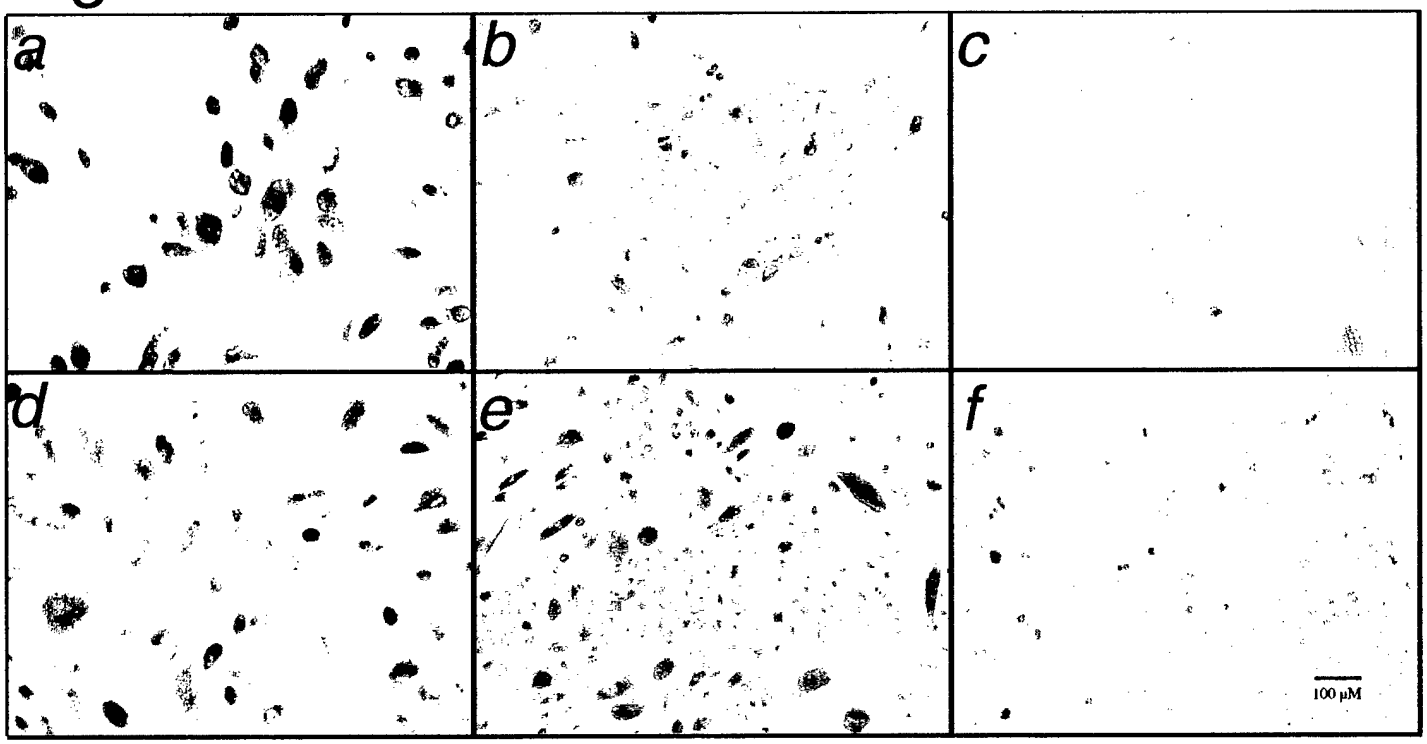
Fig.2



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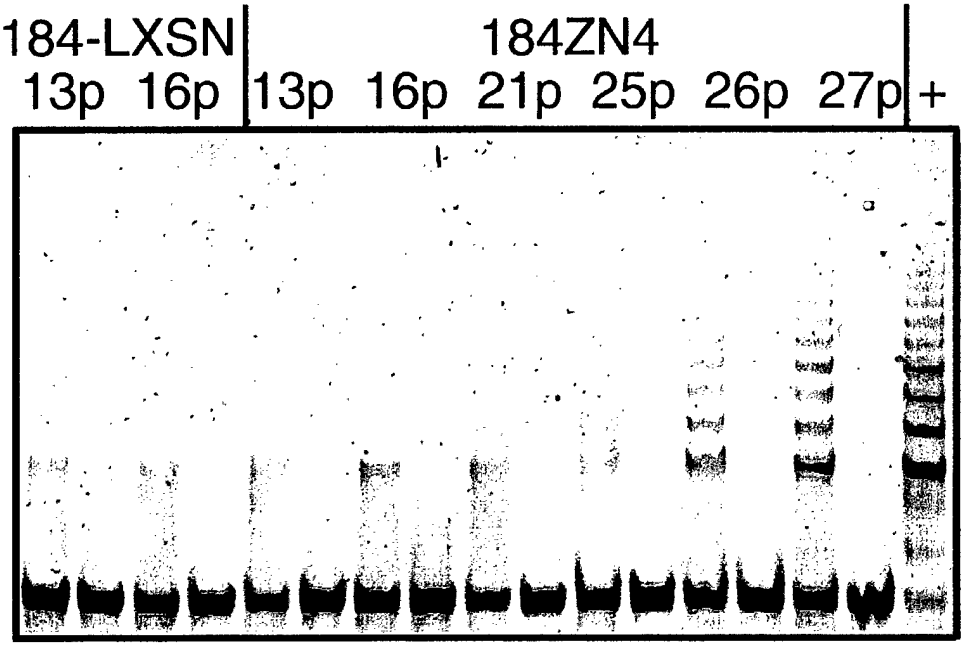
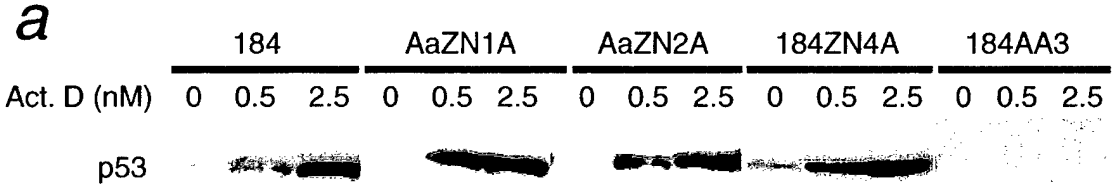

184-LXSN
184ZN4
13p 16p 13p 16p 21p 25p 26p 27p +
Heat - + - + - + - + - + - + - + - + -
Mean TRF 5.3 5.5 6.1 5.6 5.4 3.7 4.0 4.2

Fig.4

a


184
AaZN1A
AaZN2A
184ZN4A
184AA3
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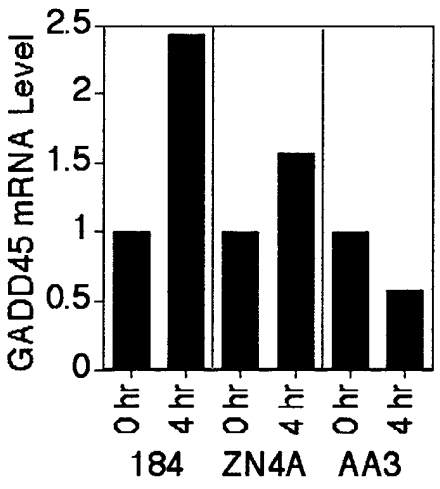
p53

b


GADD45 mRNA Level
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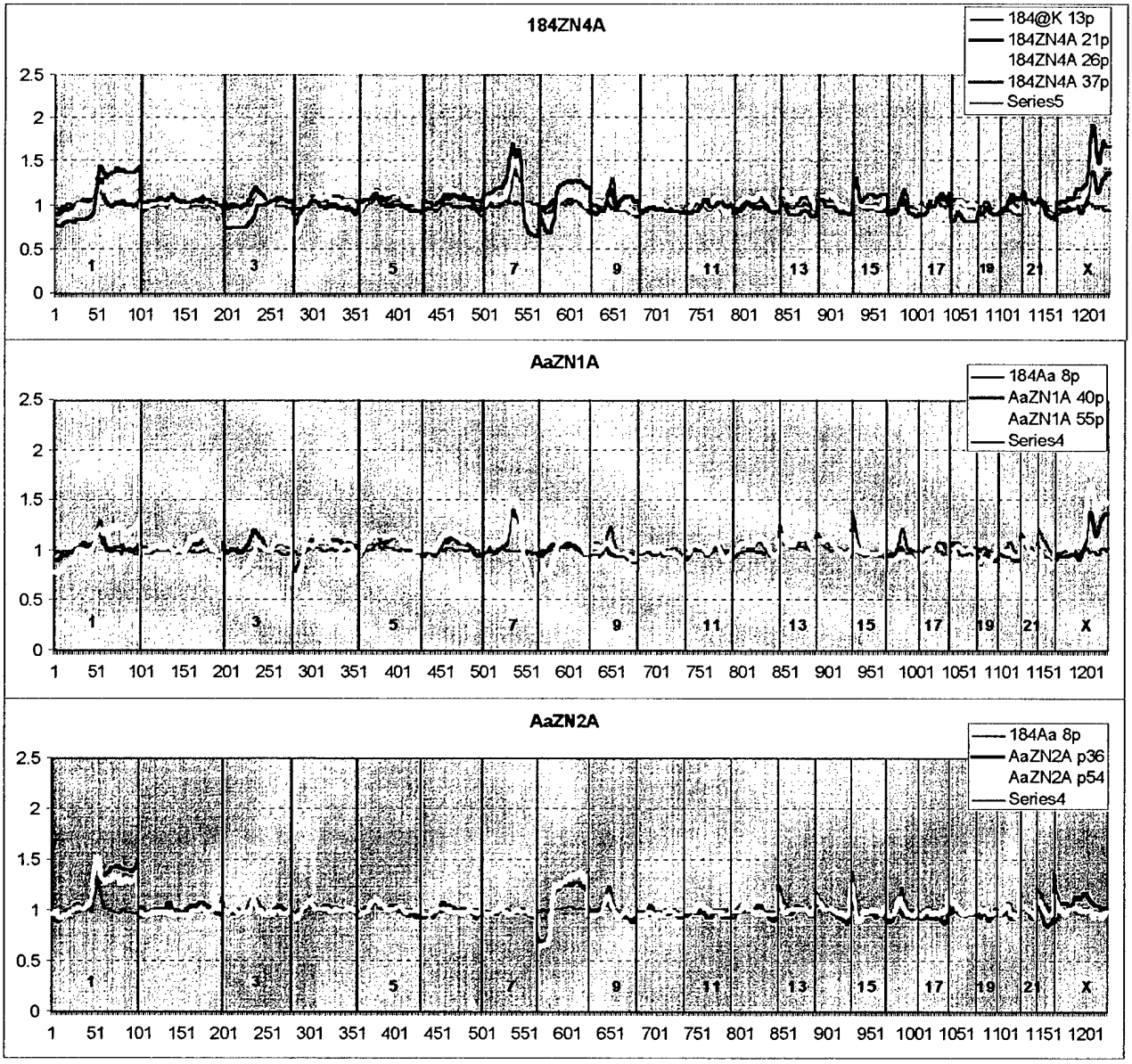
2
1.5
1
0.5
0
0 hr
4 hr
0 hr
4 hr
0 hr
4 hr
184
ZN4A
AA3

Fig.5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184ZN4A 21p
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Series5
AaZN1A
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AaZN1A 55p
Series4
AaZN2A
184Aa 8p
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AaZN2A p54
Series4
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1.5
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