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Letters to the Editor

Correspondence re: D. Zimonjic *et al.*, Derivation of Human Tumor Cells *in Vitro* without Widespread Genomic Instability. Cancer Res., *61:* 8838–8844, 2001.

Letter

We have proposed recently that aneuploidy is necessary for carcinogenesis (1–3), because it is ubiquitous in cancer (4, 5) and because it inevitably generates abnormal phenotypes by altering the expression of thousands of normal genes (6–10). This proposal was tested by Hahn *et al.* (11) and more recently by Zimonjic *et al.* (12) using three artificially mutated genes (T-antigen of SV40, a human telomerase, and the *ras* protein of Harvey sarcoma virus) to render diploid human embryo cells tumorigenic. Both studies concluded that these genes would "suffice to convert normal human cells into tumorigenic cells" and that tumorigenic cell clones did not necessarily show aneuploidy (11, 12). The more recent study was focused on subclones of tumorigenic cells selected for diploid or near-diploid karyotypes from the highly aneuploid tumorigenic HA1-ER line described by them previously (12).

As shown below, we have reanalyzed the karyotypes of the tumorigenic subclones recently selected and kindly provided to us by Zimonjic *et al.* for diploid or near-diploid karyotypes. In addition, we have analyzed independent subclones selected by us from the same aneuploid HA1-ER line obtained from Hahn *et al.* previously (11). Karyotypes were analyzed either by mFISH[1] from MetaSystems Group Incorporated using a Zeiss fluorescence microscope or by Giemsa staining, as described previously (2, 13). The chromosomal origins of hybrid chromosomes were identified via mFISH by their chromosome-specific fluorescent colors (13).

The paper by Zimonjic *et al.* (12) analyzed the karyotypes of two "near-diploid, possibly euploid" clones, HA1-ER2 and HA1-ER3, which were described as a mixture of 47–70% cells with "structurally normal, diploid complements of chromosomes" and 30–53% near-diploid aneuploid cells. These clones were karyotyped by us within 1 week after their arrival to minimize the chances of karyotype variation by culturing the cells in our laboratory. As can be seen in Table 1, all 46 cells of both clones analyzed by us were aneuploid.

The 28 cells of the HA1-ER3 clone were almost all (27 of 28) near-diploid, and all included clonal and nonclonal aneusomies and hybrid chromosomes (Table 1). For example, HA1-ER3 included the three near-clonal monosomies 7, 11, and 21 as well as partially clonal and nonclonal aneusomies. One HA1-ER3 cell was highly aneuploid with a modal chromosome number of 78 (Table 1). The hybrid chromosomes of this clone also fell into a near-clonal class, *i.e.*, 7–17, a partially clonal class including 11–20, 5–11, and even 5–13-21, a tripartite chromosome, and a nonclonal class.

The 18 cells of the HA1-ER2 clone analyzed by us fell into a near-diploid, aneuploid class (11 of 18) and into a highly aneuploid class (7 of 18) with chromosome numbers ranging from 51 to 114 (Table 1). The near-diploid class of the HA1-ER2 cells included near-clonal monosomies 11 and 14 as well as partially clonal and nonclonal aneusomies. In addition, all but one metaphase of this class (*i.e.*, number 3, Table 1) contained near-clonal, *e.g.*, 11–18 and 6–14, and nonclonal hybrid chromosomes. The highly aneuploid class of the HA1-ER2 cells carried a near-clonal disomy of the X chromosome and a near-clonal tetrasomy of chromosome 10 and many nonclonal aneusomies and nonclonal hybrid chromosomes.

We have independently derived 25 clones from single cells of the highly aneuploid HA1-ER cell line studied by us previously (2) by seeding appropriately diluted cell suspensions into plastic cloning wells. Among these clones, 20 were highly aneuploid and 5 were near diploid with modal chromosome numbers of 45 and 46. However, the individual chromosome numbers of the 5 near-diploid clones ranged from 41 to 115 (complete counts not shown). The metaphase chromosomes of one of the near-diploid clones with a modal chromosome number of 46, termed HA1-RL 96-2, were analyzed by mFISH (Table 1). All 17 cells of HA1-RL 96-2 analyzed showed evidence of aneuploidy. This included one clonal (monosomy 20) and several random aneusomies, and one clonal, 13–20, and several random hybrid chromosomes. Thus, all 25 clones obtained by us from the HA1-ER parental line consisted of either highly aneuploid or near-diploid aneuploid, but no diploid, cells.

We conclude that all of the 27 clones derived by Zimonjic *et al.* (12) and us from the tumorigenic human cell line HA1-ER are numerically aneuploid. In addition, most cells of these clones, *i.e.*, 62 of the 63 metaphases analyzed by mFISH, are also segmentally aneuploid because of hybrid chromosomes. Most hybrid chromosomes were heterodimers, presumably generated by nonreciprocal translocations of various sizes of fragments from two different chromosomes, some were homodimers and some were heterotrimers. Because there were no reciprocal counterparts of any of these hybrid chromosomes, they each contribute segmental aneuploidy to the numerical aneuploidy of these cells.

The presence of clonal aneusomies and clonal hybrid chromosomes confirmed that all cells analyzed by us were from the clonal lines provided by Zimonjic *et al.* (12) and thus excluded spurious contaminants as the source of our discrepancies. Thus, our data differ from those of Zimonjic *et al.* (12).

The discordancy between the two sets of results could be accounted for by the following:

(*a*) Zimonjic *et al.* used flow cytometry as one of two methods to identify diploid cells (12). However, because this method is insufficient to distinguish between diploid and near-diploid cells (14),[2] they might have erroneously classified near-diploid as diploid cells.

(*b*) Because the karyotypes of all cells with the SV40 T-antigen are very unstable (2), possibly preexisting, diploid cells of the clonal stocks of Zimonjic *et al.* could have been lost by the time the cells were provided to us. Indeed, the large number of nonclonal hybrid chromosomes in all HA1-ER cells analyzed here indicates that their chromosomal structure is very unstable (15). It is noteworthy that Zimonjic *et al.* (12) also reached the conclusion that "aneuploid HEK variants appear to be generated continuously . . . " even from what they described as "karyotypically normal tumor cells" (12). Moreover, their laboratory has demonstrated recently that a new set of tumorigenic human cells, prepared with the same combination of artificially mutated genes used to prepare the cells studied here, was again highly aneuploid (16). By contrast, the karyotypes of normal diploid, T-antigen free human cells are extremely stable *in vitro* (17–20).

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[1] The abbreviation used is: mFISH, multicolor *in situ* hybridization with fluorescent chromosome-specific DNA probes.

[2] Unpublished observations.

Table 1 *Karyotypes of three clonal cultures selected for diploid or near-diploid karyotypes from human cells transformed by three hypothetical cancer genes (see text).*

			Intact Chromosomes*																						Structurally Altered Chromosomes		
Clone	mt	N	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y	86-100% clonal	20-85% clonal	random
HA1-RL 96-2	1-11	46																				1					13-20		
	12	46																				1		1			13-20		2-22
	13	46				1																1					13-20		3-4
	14	50							3				3					3				1					13-20		13-18
	15	46																			3	1					13-20		
	16	45									1	1	3								1	1					13-20		11-19
	17	45																				1		1			13-20		
HA1-ER3	1	47							1														1				7-17	11-20	19-21
	2	47							1														1	1		0	7-17	11-20	21-21,22-Y
	3	46							1				1									1					7-17	11-20,5-11	
	4	45				1			1				1				1						1				7-17	5-13-21	4-11,8-15-20
	5	46							1				1											1			7-17	5-11	2-22
	6	46							1				1										1					10-11,5-13-21	
	7	47				1			1			1										3	1				7-17	16-21	d4,d8,d11
	8	46				1		1									1						1					16-21	4-6,4-6,5-15
	9	47				1			1						1							3	1				7-17	16-21	4-11,d3
	10	46							1														1	1		0		11-20	15-21,d7
	11	46					1		1				1									1	1				7-17	10-11,d5,5-13-21	2-20
	12	45							1				1									1		1			7-17	11-20,5-11	
	13	47				1			1			1				1						3	1			0	7-17	16-21	4-11,10-14,d10,d14
	14	46			1				0				1									1					7-17	5-11	3-7,3-7,11-17-20
	15	46							1				1									1						11-20,5-11	d17
	16	47				1			1													3	1				7-17	16-21	4-11-15
	17	43	1						1								1	1		1				1		0	7-17	11-20	1-15,22-Y
	18	43					1		1		1		1									1	1	1		0	7-17	d5	2-20,5-13-15-21
	19	45					1		1		1		1						1			1	1				7-17	d5,5-13-21	2-20,d9,d11
	20	45					1		1		1		0				1				1		1				7-17	10-11,d5	5-13-15,9-11,15-19,d19
	21	47					1		1				1									3	1				7-17	10-11,d5,5-13-21	
	22	44				1			1				1			1						1	1				7-17	11-20,5-11	
	23	46					1		1				1										1				7-17	10-11,d5	5-13-15
	24	48		1	1				1	1			1				1						1			0	7-17	10-11	5-15,5-8,2-10,3-8,5-Y,d13,d13
	25	46							1														1				7-17	16-21	4-11,10-14,d10,d14
	26	46							1				1									1	1				7-17	10-11,d5,5-13-21	2-20
	27	47							1											1							7-17		4-11,d18
	28	78	4	4	3	3	4	4			3	4		3	3	3	1	3	4	4	3	1	4	4	2		7-17	11-20	8-15,8-15,d7,d7,5-15-17

Table 1 *Continued*

			Intact Chromosomes*																						Structurally Altered Chromosomes		
Clone	mt	N	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y	86-100% clonal	20-85% clonal	random
HA1-	1	**44**								1			1		1					1								11-18	8-13
ER2	2	**47**		1								1	1			1												11-18,6-14	10-10,d2,d10
	3	**44**							1															1					
	4	**48**		3													1				1		3	1				11-18	3-15
	5	**46**		1					1							1					1								2-9,7-17,8-14,d19
	6	**46**											1			1												11-18,6-14	
	7	**46**								1			1			1												11-18,6-14	4-8
	8	**45**											1			0												11-18,6-14	
	9	**45**					1		1							1													4-7,4-14
	10	**46**											1			1												11-18,6-14	
	11	**114**	3	6	4	4	3	6	4		6	4		3	6	4	4		4	5	4	9	4	3	2				5-8
	12	**86**	4	4	3	1	4	4	3	3	4	4	4	3	4	4	3	3	4		4	4		4	2	2			4-4,3-12,7-16,d4,d12,d18,d18
	13	**85**	3	4	4	4	4	4	3		3	4	3	4	4	3	4	3	4		3	4		4	2	2			1-16,7-19,8-11,d8,d9,d19
	14	**79**	3	3	3	3	4	4	3		4	4	3	3	4	3	3	3	3		4	4		3	2	2			1-8,2-8,11-12,12-16-19,d17
	15	**75**	1	3			3	3	1		3	3	3	4	3	3		3	3		3	4		3	2				1-16,3-5,3-8,4-7,5-9,5-17, 7-13,7-19,8-11,d6,d6,d15,d18
	16	**80**	3	4	3	3	3	4	3		3	4	3	4		4	3	3	4		3	3			2	2			5-5,5-9,7-17,8-11,13-22, 18-19,d3,d8,d22
	17	**86**	3	5	3	3	3	4	3		3	4	3	4	4	4	3	3	4		3	3		3	2	2			1-5,3-16,4-8,5-9,7-19,8-11, 18-19,19-22,d3,d8
	18	**51**						3					3		3			3								0			2-20,5-15

* Diploid autosomes and haploid X and Y chromosomes are not recorded. Abbreviations: mt, numbers given to individual metaphases; N, number of chromosomes per metaphase; d, defective chromosomes. Hyphenated numbers identify the original chromosomes from which hybrid chromosomes were derived, e.g. 4-7 means derived from chromosomes 4 and 7.

Thus, we find significant aneuploidy in tumorigenic human cell clones that have been generated using combinations of the T-antigen, the telomerase, and the *ras* genes. This result supports, but does not prove, our hypothesis that aneuploidy is necessary for neoplastic transformation.

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Reply

In prior work, we demonstrated that it was possible to transform normal human fibroblasts and HEK[1] cells to a tumorigenic state through the introduction of three distinct genetic elements, namely the SV40 early region (which encodes both the Large T and small t antigens), the *hTERT* gene (which encodes the catalytic subunit of the telomerase holoenzyme), and a *ras* oncogene (1). Several laboratories have since reproduced these observations using many different types of human cells (2–4).

To determine whether the tumorigenic state of these fibroblasts and HEK cells required additional genetic alterations beyond those that we had introduced experimentally, we performed, among other analyses, a karyotypic analysis of these cells using both spectral karyotyping (SKY) and G-banding. Our studies revealed that all of the transformed human fibroblasts were aneuploid, whereas the transformed embryonic kidney cells consisted of two cell populations, a normal diploid fraction (70%) and aneuploid fraction (30%). We prepared single-cell clones from this polyclonal population and selected two clones (HA1ER-2 and HA1ER-3) that contained 70% or 47% diploid cells and introduced these clones into immunodeficient mice, on which occasion they formed rapidly growing tumors. When we analyzed the cells isolated from these tumors (HA1ER-2T and HA1ER-3T), we found that they, too, contained a high percentage of cells that carried a diploid complement of chromosomes (50 and 77%, respectively). We concluded that, to the limits of resolution of SKY and G-banding, a population containing a significant proportion of diploid human cells could form tumors in immunocompromised mice and that the bulk of these cells retained their diploid karyotype after having produced a tumor mass of substantial size. We further deduced that the presence of an aneuploid genome was not required for their tumorigenicity.

Since our observations were published, another group has reported that transformation of an independently derived line of human fibroblasts using introduced genes also results in transformed cells that retain a normal diploid complement of chromosomes (5).

Dr. Duesberg requested an aliquot of the original HEK cells and fibroblasts, which we provided to him shortly after our original report on the transformation of these cells was published in 1999. We provided Dr. Duesberg both tumorigenic (HA1ER and BJELR) and nontumorigenic versions (lacking *ras*, HA1EB, and BJELB) cells. In his report on these cells, his group reported that 30% of the kidney (HA1ER) and fibroblast (BJELR) cells were diploid yet concluded that aneuploidy was essential for tumorigenicity without mention of the karyotype of the nontumorigenic cells (6). As we reported (7), nontumorigenic HEK cells lacking the *ras* oncogene (HA1EB) harbored a similar population of aneuploid cells but were not tumorigenic. These observations made it highly unlikely that aneuploidy was sufficient for tumorigenicity.

In December of 2000, approximately 1 year before our report appeared in *Cancer Research*, Dr. Duesberg's group requested the cloned, transformed human embryonic kidney cells described above (HA1ER-2 and HA1ER-3). We again provided these cells, prior to our published analysis of their karyotype, with the request that he inform us of his results. Although it appears that they performed their own analysis of these cells immediately after receiving the cells, only after our report appeared in *Cancer Research* in December, 2001, did Dr. Duesberg's group provide the results of their M-Fluorescence-in-situ-hybridization (M-FISH) analysis (not a re-analysis of our own experiments), performed on cells thawed and propagated in their own laboratory. At this point, we requested that he send back to us the cells that we previously sent to him in order to confirm that they were identical to the cells that we had sent him. Two months later, these cells arrived and were then subjected to various analyses.

We have now concluded that the cells that Dr. Duesberg sent back to us were very different from the ones that we had sent him earlier. Those that he returned to us were highly aneuploid, whereas those that we sent to him contained a large percentage of diploid cells. On close inspection, it appears that some but not all of the cells that constituted the minority aneuploid fraction of our original cells have been amplified in the cell populations reported in Dr. Duesberg's letter (*e.g.*, HA1ER-3 metaphases with a deletion of chromosome 7). Our own observations indicate that under the conditions we described, including the formation of tumors in animals, that a high percentage of diploid cells persists.

We can only conclude that it is possible, as has long been known, that the improper handling of cultured cells, including their thawing and culturing under suboptimal conditions, can encourage and select for the outgrowth of aneuploid variants, and that Dr. Duesberg's observations once again provide testimonial to this long-accepted principle. We stand by our initial observations and do not find any reason to re-interpret them or to qualify our conclusions on the basis of the observations described by Dr. Duesberg.

Dr. Duesberg has consistently ignored a large body of literature supporting a causal role for oncogenes and tumor suppressor genes in the genesis of cancer. In addition, by ignoring internationally recognized cytogenetic nomenclature to describe their karyotypic analysis, Dr. Duesberg and his colleagues needlessly obscure their results. From his letter, we understand his present argument, that specific types of chromosomal

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[1] The abbreviation used is: HEK, human embryonic kidney.

abnormalities accompany distinct stages of cell transformation, to support the widely held view that such karyotypic abnormalities harbor alterations in certain genes, specifically those that program the malignant state. This would lead one to conclude, as we have also argued, that aneuploidy facilitates the acquisition of the genetic alterations that lead to cancer. It does not, however, provide any indications that aneuploidy is an essential cause of malignant cell transformation. Instead, aneuploidy is only one of several means by which normal cells can acquire the spectrum of genetic alterations that is needed to enable their growth as cancer cells.

Finally, we note that the bulk of the observations that are presented in tabular form in Dr. Duesberg's letter are identical to those produced by and provided to us previously (January 2002) by Dr. Stindl, a postdoctoral researcher who has since left the Duesberg laboratory but who does not appear as a cosignatory on this letter from his group.

As we indicated in our report published in this journal, we conclude that mechanisms that generate genomic instability and result in aneuploidy play an important role in spontaneously arising cancers by facilitating the accumulation of genetic mutations that program the cancer phenotype; such genetic instability and aneuploidy are obviated by providing cells, as we have done, with a set of genes that specify the full complement of cell phenotypes required for tumorigenic growth. Our observations do not completely eliminate the possibility that other subtle mutations exist in these experimentally transformed human cells. However, the karyotypic and functional experiments presented in our report make it highly likely that the observed tumorigenicity of these cells derived directly from the introduced genes rather than from indirect effects created by the karyotype of these cells.

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