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Heterogeneity of Lineage Involvement by Trisomy 8 in Myelodysplastic Syndrome

A Multiparameter Analysis Combining Conventional Cytogenetics, DNA In Situ Hybridization, and Bone Marrow Culture Studies

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ABSTRACT: *To better understand the role of trisomy 8 in myelodysplastic syndrome (MDS), we performed a multiparameter analysis combining conventional chromosome studies (CCS), fluorescence in situ hybridization (FISH), and bone marrow (BM) culture studies in two patients with MDS evolving into acute myeloid leukemia (AML).*

A mosaicism of a cytogenetically normal clone and a clone with trisomy 8 was detected in both patients throughout the course of the disease, a finding confirmed by FISH on BM cells. The relative size of the trisomic clone increased from 52% to 71% ($p < 0.0001$) and from 53% to 69% ($p = 0.001$) of all BM cells at the time of the leukemic switch in patients 1 and 2, respectively.

Combined FISH and immunophenotyping of BM cells showed involvement of the granulomonocytic lineage in patient 1 and involvement of erythroid cells as well as of the granulomonocytic lineage in patient 2. Only disomic lymphocytes were detected in both patients.

FISH on single hemopoietic colonies grown in semisolid media detected trisomic CFU-GM and disomic BFU-E in patient 1, whereas a proportion of CFU-GM and BFU-E deriving from the trisomic clone was detected in patient 2. However, the percent of trisomic colonies was lower than the percent of involved granulomonocyte precursors and involved erythroblasts, as detected by combined FISH and immunophenotyping on fresh BM samples.

We have thus shown heterogeneity of lineage involvement by trisomy 8 in MDS undergoing transformation into AML. Although preferential growth of disomic clones may occur in vitro, the finding of an increased size of the trisomic clone at the time of leukemic switch suggests that these cells had proliferative advantage in vivo over cells without trisomy 8.

INTRODUCTION

A number of non-random chromosome changes have been described in myelodysplastic syndrome (MDS) [1], reflecting the heterogeneity of the clinicobiologic features of this myeloid neoplasia characterized by ineffective hemopoiesis resulting in progressive cytopenia affecting one or more cell lineages. Trisomy 8 is one of the most frequently occurring numerical change in MDS, and does not appear to be restricted to any of the cytologic subsets identified by the FAB group in 1982 [2].

Cytogenetic studies of different cell populations obtained by separation over a density gradient, as well as chromosome analysis of single hemopoietic colonies, documented that trisomy 8 may involve both the erythroid series and the granulomonocytic lineage [3, 4]. However, recent investigations combining fluorescence in situ hybridization (FISH) and membrane immunophenotyping to detect numerical aberrations in interphase nuclei documented the heterogeneity of distribution of this chromosome anomaly in the different hemopoietic lineages [5, 6].

Consequently, the biologic significance of trisomy 8 in MDS remains poorly understood. We therefore designed a study combining conventional chromosome analysis, FISH, and immunophenotyping, as well as bone marrow culture studies, in two patients with MDS evolving into acute myeloid leukemia (AML) with trisomy 8, to analyze the distribution of this numerical aberration in different hemopoietic

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lineages and to better understand its possible role in the natural history of MDS undergoing transformation into AML.

PATIENTS AND METHODS

Two patients with MDS evolving into AML carrying trisomy 8 were studied. Diagnoses were made according to the FAB criteria [2, 7]. Cytogenetic, FISH studies, and in vitro assays of progenitor cell growth were performed as summarized below.

Patient 1

This 65-year-old patient was referred to our institute in January 1992 for anemia and splenomegaly (4 cm below left costal margin). The blood count revealed hemoglobin (Hb) 8 g/dL, platelet (Plt) 40 × 10^9/L, white blood cell (WBC) 2 × 10^9/L, with 3% blasts. The BM was hypercellular, with hyperplasia of the granulomonocytic lineage and relative reduction of the erythroid series (see Table 1). Nuclear as well cytoplasmic dyserythropoiesis was apparent in 20% erythroblasts. The majority of neutrophils were hypogranular. Megakaryocytes were reduced and a moderate increase of lymphocytes and plasma cells was observed. Based on the presence of 27% blasts in the BM, a diagnosis of refractory anemia with excess of blasts in transformation (RAEB-t) was made.

The patient required intensive supportive therapy throughout the course of the disease. Progressive reduction of mature forms of the granulocytic lineage concomitant with a gradual increase of blast cells was recorded over a 2-month period, resulting in leukemic switch with 45% BM blast cells with myelomonocytic features.

Therapy with cytosine arabinoside (Ara-C) 10 mg/m^2, by intravenous infusion, twice a day for 10–14 days was given. Six courses were administered between May 1992 and April 1993, obtaining only partial short-lasting reduction in the number of blast cells. Progressive spleen enlargement developed and the patient died 18 months after diagnosis with disease progression.

Patient 2

This 67-year-old patient was admitted to our institute in February 1992 for petechiae of the pelvic limbs. The blood count revealed pancytopenia: Hb 8.5 g/dL, Plt 50 × 10^9/L, WBC 3 × 10^9/L, with 18% granulocytes, 76% lymphocytes, 4% monocytes, and 2% blasts. The bone marrow sample, hypercellular with trilineage myelodysplasia, revealed 24% blasts. These findings were compatible with a diagnosis of RAEB-t.

Progressive leftward shift of granulocytopoiesis with increase of BM blast cells to 38% was observed during a 3-month period. Blast cells were occasionally granular and showed Sudan black-B positivity. These findings were consistent with a transformation of MDS into AML without maturation. Treatment with Ara-C, 10 mg/m^2, by intravenous infusion, twice a day for 7–10 days was administered. Eleven courses of low-dose Ara-C were given between March 1992 and October 1993, obtaining a reduction in the number of BM blast cells. The patient is presently alive and well, with a cytologic picture consistent with RAEB.

Colony Assay

In vitro growth of erythroid colonies (BFU-E) and of granulomonocytic colonies (CFU-GM) was assayed according to the methods described by Iscove et al. [8, 9]. In the BFU-E growth assay erythropoietin (Boehringer Mannheim) was added to give a final concentration of 2 U/ml. Recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF, 10 ng/mL, Boehringer Mannheim, Germany) was used to stimulate growth of CFU-GM.

Chromosome Studies on Bone Marrow Cells

Bone marrow cells were cultured for 24 and 48 hours without mitogens. Synchronization with methotrexate and thymidine was performed. Metaphases were G-banded with Wright's stain. Chromosome aberrations were described according to the International System for Human Cytogenetic Nomenclature [10].

Cytogenetic Studies of Individual CFU-GM and BFU-E

To analyze the karyotype of the progeny of single hemopoietic colonies grown in semisolid media and to assess the number of BFU-E and CFU-GM with and without trisomy 8, conventional cytogenetic as well as FISH studies of hemopoietic colonies were performed. Chromosome analysis from individual colonies were performed on days 8 to 10 for CFU-GM and on days 12 to 13 for BFU-E. Colcemid (100 μL of a

Table 1 BM, cytogenetic, and interphase cytogenetic findings in two patients with MDS evolving into AML

Patient, sex/age (yr)	% Bls/% GM cells/ % Ebls (BM)[a]	Diagnosis	BM karyotype	No. abnormal/total no. metaphases	No. of fluorescent spots in interphase cells[b]		
					1	2	3
1, M/65	27/53/11	RAEB-t	47,XY, + 8	25/43	6	42	52
	45/43/10	AML-M4	47,XY, + 8	33/39	8	21	71
2, M/67	24/54/26	RAEB-t	47,Y,der(X)t(X;1)(q27;q25), + 8	16/23	6	41	53
	38/47/23	AML-M1	47,Y,der(X)t(X;1)(q27;q25), + 8	19/21	4	27	69

Abbreviations: Bls, blast cells; GM cells, cells of the granulomonocytic lineage; Ebls, erythroblasts.

[a] Differential on 300 BM cells; other cells were megakaryocytes, lymphocytes, and plasma cells.

[b] 200 cells with well-delineated signals observed. Less than 1% cells with three spots were detected in a control slide from a normal subject.

0.1 μg/mL solution) was added to cultures 6 hours prior to the end of the incubation period. Single colonies were individually aspirated and transferred into an Eppendorf tube containing 80 μL of 0.075 KCL. After incubation (30 minutes, 37°C), the entire content of the Eppendorf tube was transferred onto a microscope slide pre-treated with poly-L-lysine. The slides were incubated (30 minutes) in a moist chamber. Methanol/acetic acid (3:1) fixative was dropped onto the area of the slides where the colony was placed. The metaphases suitable for analysis were G-banded with Wright stain and karyotyped.

The same procedure was adopted to prepare slides from single hemopoietic colonies for the purpose of FISH study (see below).

Fluorescence In Situ Hybridization

For the detection of trisomy 8 in interphase cells from BM samples and from single hemopoietic colonies, the chromosome 8-specific centromeric probe (Oncor Inc., Gaithersburg, MD) was used. The percentage of interphase cells with trisomy 8 was assessed by FISH on BM cells at diagnosis and at leukemic switch in both patients. Slides prepared from BM samples for conventional cytogenetic analysis, aged at room temperature for 5–15 days, were treated with RNAse (RNAse A, 100 μg/mL in 2 × SSC), dehydrated in alcohol, and denatured in 70% formamide at 70°C for 2 minutes. The biotinylated probe (100 ng/slide) was dissolved in 45 μL hybridization mixture (total volume) consisting of 60% formamide in 2 × SSC, 10%wt/vol dextran sulphate, and 30 μg salmon sperm DNA.

Following denaturation at 75°C for 5 minutes, 45 μL of hybridization mixture were layered onto each slide. The slides were covered with a 24 × 50 mm coverslip and sealed with rubber cement (Sanford, Bellwood). Hybridization was allowed to proceed overnight at 37°C in a moist chamber. The slides were then washed in four changes of 50% formamide at 45°C for 3 minutes and in four changes of 2 × SSC at 45°C for 3 minutes. Following a 20-minute blocking step with 5% non-fat dry milk powder in 2 × SSC, the slides were incubated with FITC-avidin (Vector, Burlingame, CA) (5 μg/mL in 4 × SSC/5% non-fat dry milk) for 20 minutes.

Signal amplification was obtained with a layer of biotinylated anti-avidin monoclonal antibody (Vector, Burlingame; 5 μg/mL), followed by incubation with FITC-avidin. Propidium iodide (0.5 μg/mL) was used as a counterstain. Slides were examined under a fluorescence microscope (Diaplan Leitz); 200 BM interphase cells with well-delineated fluorescent spots were counted in each patient for signal screening. At least 50 interphase cells from each single hemopoietic colony were observed.

Combined Immunophenotyping and FISH

To determine the percentage of erythroblasts, granulocyte precursors, and lymphocytes carrying trisomy 8, combined immunophenotype/FISH study was performed on BM cells at diagnosis.

Following separation over a Ficoll gradient, the mononuclear cells were incubated with the following monoclonal antibodies: anti-CD13 (My7, Coulter Inc.) for granulocytic lineage, anti-CD14 (My4, Coulter Inc., Hialeah, FL) for mature monocytic cells, anti-glycophorin (OKRBC, Ortho Diagnostic System Inc., Raritan, NJ) for erythroid lineage, anti-CD2 (T11, Dakopatts, Copenhagen, Denmark) labeling T lymphocytes, and anti-CD19 (Leu12, Becton-Dickinson, Mountain View, CA) labeling B lymphocytes. To minimize non-specific Fc binding, cell suspensions were preincubated with 5% normal rabbit serum (Dakopatts).

Specific binding of the primary antibody was visualized with rabbit anti-mouse immunoglobulin conjugated with tetramethyl rhodamine isothiocyanate (TRITC) (Dakopatts). Cytospin preparations were made from each immunolabeled cell suspension and fixed by immersion for 30 minutes in methanol/acetic acid at a concentration 19:1 and by a second immersion for 15 minutes in methanol/acetic acid at a concentration 3:1.

The same hybridization protocol as described above was employed for the detection of trisomy 8 in immunolabeled cells. No counterstaining was used. At least 100 immunolabeled cells with well-delineated signals were observed for each monoclonal antibody employed.

RESULTS

Cytogenetic and interphase cytogenetic findings are described in Table 1. Results of combined innunophenotyping and DNA in situ hybridization and FISH on single colonies are presented in Tables 2 and 3.

Chromosome studies on single colonies yielded 2–7 (me-

Table 2 Progenitor assays and cytogenetic studies on individual colonies in two patients with RAEB-t

Patient diagnosis	Progenitor assays[a]		Karyotype of individual colonies[b]			
	CFU-GM	BFU-E	CFU-GM		BFU-E	
1 RAEB-t	90	42	47,XY, + 8	(4)	46,XY	(20)
			46,XY	(16)		
2 RAEB-t	105	20	47,Y,der(X), + 8	(3)	47,XY,der(X), + 8	(1)
			46,XY	(15)	46,XY	(19)

[a] Each value shows the mean of quadruplicate cultures. Normal value for CFU-GM: 120–200/10^5 BM mononuclear cells. Normal value for BFU-E, 70–120/10^5 BM cells.

[b] Conventional cytogenetic analysis, number of colonies in parentheses.

dian 4) analyzable metaphases from each colony. The same karyotype was present in all metaphases obtained from a single colony. Salient results in each patient are summarized below.

Patient 1

Trisomy 8 was present in 25/43 of analyzed metaphases at diagnosis, a figure confirmed by FISH studies revealing 52% interphase cells with three fluorescent signals (Table 1). The relative size of the BM trisomic clone was found to be increased by FISH to 71% of all interphase cells at leukemic switch ($p < 0.0001$), when 33/39 metaphases with +8 were obtained (see Table 1).

Combined immunologic and FISH analyses showed three fluorescent signals in 49% CD13+ cells and in 39% CD14+ cells. Conversely, no positive cell with markers for the lymphocyte and erythroid lineage displayed three fluorescent signals. Cytogenetic analysis on single colonies showed the presence of 4/20 (20%) CFU-GM with trisomy 8, while this aberration was not present in any of the BFU-E cells examined. Similar findings were obtained by FISH on the single colonies, as shown in detail in Table 3.

Patient 2

A 47,Y,der(X)t(X;1)(q27;q25), +8, karyotype was detected in 16 of the 23 metaphases analyzed from BM samples at diagnosis. FISH analysis revealed the presence of three fluorescent signals in 53% of the bone marrow cells. Trisomic cells increased to 69% in the leukemic phase ($p = 0.001$), when 19/21 metaphases showed the same clonal abnormalities detected at diagnosis (see Table 1). Combined immunologic and FISH analysis (Fig. 1) demonstrated the presence of trisomy 8 in 62% CD13+ cells, in 27% CD14+ cells, and in 21% OKRBC+ cells. T and B lymphocytes did not have evidence of trisomy 8.

Cytogenetic studies on single colonies revealed the presence of the 47,Y,der(X)t(X;1)(q27;q25), +8, karyotype in 3/18 (16.6%) of the CFU-GM, a finding confirmed by FISH analysis showing 4/20 trisomic CFU-GM. Evidence of trisomy 8 in 1/10 and in 1/20 individual BFU-E was provided by FISH and by conventional chromosome analysis, respectively. Detailed results are summarized in Tables 2 and 3.

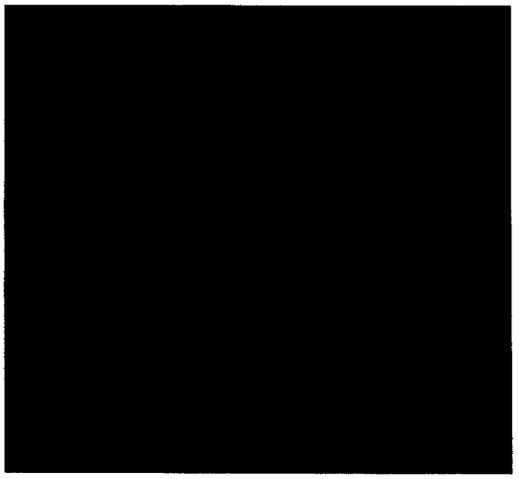

Figure 1 Combined FISH and immunophenotyping on BM cells from patient 2: a CD13+ cell with three fluorescent signals using a chromosome 8-specific probe is shown.

DISCUSSION

Although trisomy 8 is one of the most frequently occurring chromosome change in MDS, its role in the natural history of this primitive hemopoietic stem cell disorder remains elusive. Indeed, while debate still surrounds its possible prognostic significance [11, 12], reports showing multilineage involvement by trisomy 8 in MDS with progressive accumulation of the trisomic clone in the BM have been published [5, 6, 13, 14], along with studies documenting that fluctuations of the size of the trisomic clone may occur rather frequently without correlation with disease evolution [15, 16].

By circumventing the need for metaphases, FISH enables cytogenetic study of adequate numbers of interphase cells, thus permitting the estimation of the clone size as well as the assessment of the distribution of chromosome aberrations in immunolabeled cells [17, 18]. In addition, the study of hemopoietic colonies grown in semisolid media can be eas-

Table 3 Comparison of combined FISH and immunophenotyping with FISH on single hemopoietic colonies to assess lineage involvement in two patients with RAEB-t

Monoclonal antibody tested	Signal screening in immunolabelled cells[a]			FISH on individual colonies[b]	
	1	2	3	No. of CFU-GM with three spots/ total no. assessed	No. of BFU-E with three spots/ total no. assessed
Patient 1					
Anti CD13	10	41	49	5/20	0/12
Anti CD14	8	53	39		
Anti-glycophorine	9	91	0		
Patient 2					
Anti CD13	9	29	62	4/20	1/10
Anti CD14	6	67	27		
Anti-glycophorine	10	69	21		

[a] No CD2-positive and CD19-positive cells with three signals were detected (100 cells observed).

[b] More than 80% cells with three signals were found in each colony classified as trisomic; more than 80% cells with two signals were found in each colony classified as disomic.

ily performed by FISH, allowing for the comparison of the percent of trisomic cells in the in vitro culture system usually adopted for colony assays and in uncultured BM samples.

Because few patients with MDS and trisomy 8 have been studied, we designed this multiparameter analysis of the distribution of trisomy 8 in erythroid, granulomonocytic, and lymphoid lineage in two patients with MDS evolving into AML. Discussion will focus on the issue of lineage involvement by trisomy 8 in these patients apparently running the same clinical course, and on the comparative analysis of the percentage of trisomic cells in BM cells identified immunologically, as well as in single CFU-GM and BFU-E.

Both disomic and trisomic cells were detected in BM cells in our patients by conventional chromosome analysis and by FISH, showing a fairly good correspondence between the percentage of metaphase cells with trisomy 8 and the percentage of interphase nuclei with three fluorescent signals. This finding confirms that FISH allows for the reliable detection of trisomy 8 in interphase cells in MDS [19]. It is worth noting, however, that caution should be employed when interpreting these data since errors in assessing the abnormal-to-normal metaphase ratio may occur frequently [12]. In addition, the FISH artefact rate has been shown recently to be relatively high, suggesting the need for an automated signal evaluation [20].

In view of the mosaicism of the disomic and trisomic clone in BM samples, it was of interest to assess which cell population carried trisomy 8 in these patients. B and T lymphocytes in our patient did not carry trisomy 8, as shown by combined FISH and immunophenotyping. This finding is in keeping with previous reports consistently showing that chromosome aberrations in MDS are usually restricted to the nonlymphoid lineage [21–23]. However, molecular genetic studies of the nature of lymphopoiesis in MDS have shown that lymphocytes may be part of the clonal proliferation in a fraction of MDS [24, 25], raising the possibility that clonal hemopoiesis may precede the development of chromosome aberrations in some patients [26].

Data obtained by combined immunophenotyping and FISH in this study provide evidence for the involvement of the granulomonocytic lineage by trisomy 8 in our patients. By contrast, erythroid cells carrying trisomy 8 were only seen in patient 2, trisomic erythroblasts not having been detected by double FISH and immunophenotyping in patient 1 in the present study. Different hypothetical scenarios may be envisaged to account for the heterogeneity of lineage involvement by trisomy 8 in our two patients.

Trisomy 8 may represent a clonal abnormality affecting a stem cell capable of differentiating along the granulomonocytic pathway only, acquired during the evolutive phases of MDS. In patient 1, it is reasonable to assume that the clonal proliferation leading to refractory anemia was initially cytogenetically normal and that trisomy 8 was acquired when progressive accumulation of blast cells, later leading to overt leukemia, started to occur. This interpretation would explain why the erythroid lineage did not carry trisomy 8 and is in keeping with the evolution pattern of MDS previously described by Tricot et al. [27].

Alternatively, trisomy 8 may be acquired in the early stages of the natural history of MDS and may involve a multipotent stem cell giving rise to a progeny of erythroblasts and granulocytes. Data obtained from patient 2 suggest that the trisomic clone may coexist with cytogenetically normal progenitors and that disease progression may occur, possibly due to a proliferative advantage of this chromosomally abnormal clone. Indeed, a statistically significant increase in the percentage of trisomic cells in BM samples was detected by FISH in our two patients when overt leukemia developed.

The above considerations, along with the analysis of the clinical course in our two patients, which proved to be rather aggressive in patient 1 and indolent in patient 2, raise the issue of clinicobiologic heterogeneity of RAEB-t, a subset of MDS possibly including cases of "early AML," as well as genuine cases of preleukemia [28].

Consistent involvement of granulocyte precursors and erythroblasts by trisomy 8 in MDS was reported in previous studies either using chromosome analysis of different cell populations obtained by separation on a density gradient [4], cytogenetic studies on progenitor cells [3], or chromosomal in situ suppression hybridization [13].

Two additional cases with trisomy 8 in cells of the granulomonocytic and erythroid lineage were recently found by combined immunophenotyping and DNA in situ hybridization [5, 6]. However, one patient with trisomy 8 that was confined to the erythroid lineage was also described [5], further increasing heterogeneity of lineage involvement by this chromosome aberration in MDS.

In vitro growth of hemopoietic precursors is usually impaired in MDS, reflecting disordered in vivo proliferation. Several mechanisms are likely to be involved in the genesis of this abnormal growth and the effect of clonal chromosome changes is unclear. Therefore, the presence of a mosaicism of a disomic and trisomic clone in our two cases prompted us to assess whether colonies growing in vitro originated from chromosomally normal cells or from trisomic progenitor cells.

Conventional cytogenetic studies on single BFU-E and CFU-GM revealed that a proportion of erythroid and myeloid colonies originated from the cytogenetically abnormal stem cell in patient 2, in whom the involvement of erythroblasts and myelomonocytic cells was previously demonstrated by combined FISH and immunophenotyping.

In line with data deriving from combined immunophenotyping and FISH, some trisomic CFU-GM and only cytogenetically normal BFU-E were detected in patient 1. In both patients, no additional clones with chromosome changes other than those found in direct cytogenetic harvest of BM samples were detected in progenitor cells grown in semisolid medium. This culture system, therefore, does not appear to favor the preferential in vitro growth of latent subclones with unrelated karyotypes, which have been shown recently to be detectable in MDS patients by a long-term BM culture system [29]. This finding is in keeping with a previous report [30].

For a better estimate of the number of trisomic progenitor cells growing in vitro in the MDS phase, we performed FISH on single colonies. In patient 2, the percentage of BFU-E and CFU-GM with trisomy 8 was lower than the percentage of erythroblasts and granulocyte precursors showing three fluorescent signals at combined immunologic/FISH analysis of

BM cells. Likewise, preferential growth of cytogenetically normal CFU-GM was demonstrated in patient 1.

Therefore, this culture system may have facilitated the growth and differentiation of chromosomally normal progenitor cells over trisomic cells. Potentially, modifications of the routine culture system adopted in this study may be capable of further enhancing proliferation of chromosomally normal progenitors. Swolin et al. [30] found in two MDS patients with a 5q- chromosome and with monosomy 7 a significantly lower proportion of chromosomally abnormal CFU-GM with respect to the proportion of abnormal metaphases in a direct BM preparation. Unlike our cases, a clone with trisomy 8 in a patient with MDS appeared to have growth advantage in vitro in comparison with normal clones. In this study, however, no information is provided concerning which cell lineage was involved by trisomy 8, making comparison with our data difficult.

In conclusion, we have shown heterogeneity of lineage involvement by trisomy 8 in two patients with MDS employing a combined immunophenotyping and DNA in situ hybridization technique and cytogenetic study of progenitor cells grown in semisolid media. Although preferential growth of chromosomally normal progenitor cells may occur in vitro, the relative size of the trisomic clone increased at the time of leukemic switch, suggesting that a positive selective pressure allowed for the establishment and expansion of trisomic cells in vivo.

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