24-EPIBRASSINOLIDE AT SUBNANOMOLAR CONCENTRATIONS MODULATES GROWTH AND PRODUCTION CHARACTERISTICS OF A MOUSE HYBRIDOMA+

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Brassinosteroids are known to stimulate plant growth and to possess antistress activities in plants. This work was aimed at exploring possible beneficial effects of 24-epibrassinolide on cultured mammalian cells. A mouse hybridoma was cultured either in standard serum-free medium, or in medium diluted to 30%, in which the cells underwent nutritional stress. Steady-state parameters of semicontinuous cultures conducted at 24-epibrassinolide concentrations from 10^{-16} to 10^{-9} mol l^{-1} were evaluated. Typical effects of the agent found both in standard and in diluted media were (i) increase in the value of mitochondrial membrane potential, (ii), drop of intracellular antibody level, (iii) increase in the fraction of the cells in the G_0/G_1 phase, and (iv) decrease in the fraction of the cells in the S phase. Alleviation of nutritional stress manifested itself in cultures conducted in diluted media. Viable cell density was significantly higher (relative to control) at 24-epibrassinolide concentrations 10^{-13} and 10^{-12} mol l^{-1}. The results of this exploratory study show that the plant hormone 24-epibrassinolide may induce perturbations in the cell division mechanism, in mitochondria performance, and in secreted protein synthesis in a mammalian cell line. At the lowest brassinosteroid concentrations, the number of steroid molecules in the culture was of the same order of magnitude as the number of viable cells in the culture. This implies involvement of a complex cascade mechanism, through which the steroid molecule induces alterations in gene expression leading finally to significant changes in cell culture parameters.

Keywords: Steroids; Brassinosteroids; 24-Epibrassinolides; Hybridoma cells; Mitochondrial membrane potential; Cell cycle; Monoclonal antibodies; Plant growth regulators.

+ Part CDXVI in the Series On Steroids; Part CDXV see ref.[1]

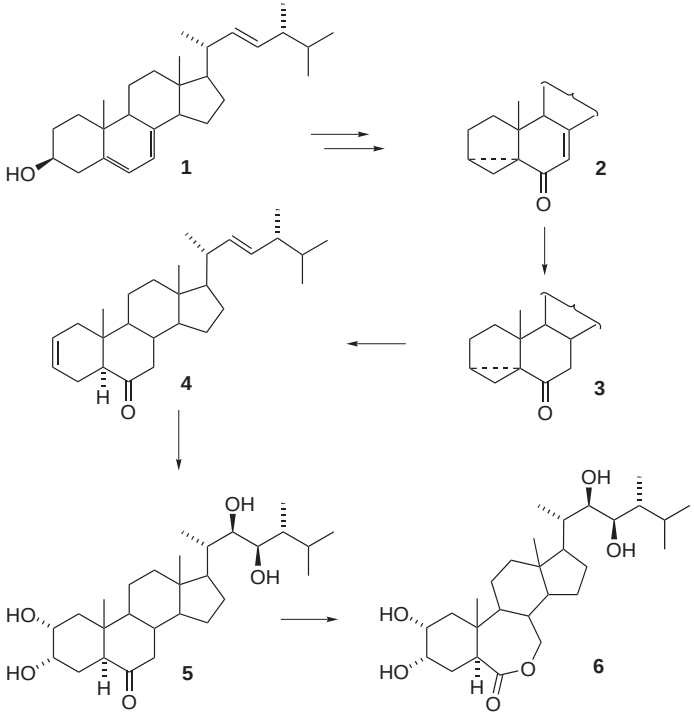
Brassinosteroids are a group of phytohormones, like auxins, cytokinins, gibberellins and abscisic acid. These compounds are widespread in nature, and can be found in higher and lower plants, gymnosperms, monocotyledons, dicotyledons and algae. Being present in almost every part of the plant, their highest concentration is in pollen (10^{-5} or less per cent), and high concentration in seeds. The term brassinolide is derived from *Brassica napus* L., from the pollen of which they were isolated for the first time[2]. Brassinolide, *i.e.* (22*R*,23*R*,24*S*)-2α,3α,22,23-tetrahydroxy-24-methyl-7-oxa-7a-homo-α-cholestan-6-one, is the main representative.

As the distribution of brassinosteroids in plant material is very low and their isolation from this source affords amounts, insufficient for practical use, many brassinosteroids with higher or lower brassinolide activity were synthesized. Some of the synthetically prepared brassinosteroids were later found to occur in nature. An example of such compound, synthetically more easily available in comparison with brassinolide, is its 24-epimer, 24-epibrassinolide, (22*R*,23*R*,24*R*)-2α,3α,22,23-tetrahydroxy-24-methyl-7-oxa-7a-homo-α-cholestan-6-one (hereafter epibrassinolide or compound **6**). Its synthesis[3] was described in 1979, and isolation[4] from green algae in 1987. Due to a difficult availability of brassinolide, most studies and practical applications utilize 24-epibrassinolide, which has almost the same biological activity as brassinolide. 24-Epibrassinolide was also used in this work. The terms brassinolide and epibrassinolide are sometimes interchanged in literature, which can lead to serious misunderstanding. Therefore, it is necessary to follow exactly the correct use of these names[5].

The best known physiological activity of brassinosteroids is their ability to stimulate plant growth[6]. They cause elongation of all parts of plants, of stems, shoots, roots, the quantity of buds, quantity and weight of pods, they also act in cell division, influence seed germination, *etc.* Elongation, swelling and splitting of young beans were observed when brassinosteroids were applied at the beginning of the second internode growth[7]. Especially interesting are their antistress activities, *i.e.*, against temperature stress, water stress, salt stress and chilling stress, which are now very intensely studied. For example, the protection of cucumber plants against heat stress (40 °C) after treatment with brassinolide was found to result in an increase of germination ability, better growth and higher dry weight[8]. A decrease of the effect of water stress in mung beans treated with brassinolide was shown to be associated with a higher ability of plants to assimilate water[9]. The use of brassinosteroids for the elimination of stress in plant development was also reported in patent literature[10].

Some effects of brassinosteroids were observed in vertebrates as well. Treatment of Russian sturgeon *Acipenser gueldenstaedti* fingerlings with 10^{-4} mg l^{-1} epibrassinolide before exposure showed a significant decrease of the negative influence of stress factors, such as pollutants, oxygen deficiency and high salinity[11]. Treatment of several *Acipenser* species at 10^{-5} to 10^{-9} mg l^{-1} with epibrassinolide increased fecundity, hatching and larvae survival. The authors also reported a significantly increased viability of spermatozoa in sperm treated with epibrassinolide at 10^{-6} mg l^{-1}.

The aim of this work was to examine the activity of epibrassinolide in mammalian cells transformed to permanent growth *in vitro*. A model of lymphocyte hybridomas growing in chemically defined media free of steroids and any natural macromolecules offered a possibility to study the effect of epibrassinolide without interference caused by ill-defined natural materials. To our knowledge, this is the first study devoted to exploring the effects of brassinosteroids in a mammalian cell system.



SCHEME 1

EXPERIMENTAL

24-Epibrassinolide Synthesis

The synthesis of 24-epibrassinolide used in this work is a modification of previously described syntheses, resulting in a higher yield of the substance (Scheme 1). The starting material for the synthesis was ergosterol (Fluka, purum; >97% (HPLC)), *i.e.* 3-hydroxy-24-methylcholest-5,7,22-triene (**1**). In 3α,5-cyclo-6-oxo-7-ene (**2**), available from ergosterol *via i*-steroid rearrangement and oxidation, the 7-double bond was reduced with $Na_2S_2O_4$ in the presence of a phase transfer catalyst[12,13]. The obtained 3,5-cyclo-6-oxo-24-methylcholest-22-ene (**3**) was then converted to 6-oxo-2-olefine (**4**). Compound **3** was isomerized in dimethylacetamide with lithium bromide and pyridinium 4-toluenesulfonate[14]. Improvement of hydroxylation[15] led to the more desired 22*R*,23*R*-isomer **5** upon asymmetric dihydroxylation with *O*-(4-chlorobenzyl)hydroquinidine (Fluka; >99% (HPLC)). In this step, we obtained 24-epicastasterone [(22*R*,23*R*,24*R*)-2α,3α,22,23-tetrahydroxy-24-methyl-5α-cholestan-6-one; (**5**)]. The conversion of 24-epicastasterone **5** into 24-epibrassinolide **6** was effected by Baeyer–Villiger oxidation. We improved this part of the synthesis[16] by excluding the steps of protection and deprotection of the hydroxyl groups in positions 2,3,22,23. Hence, we obtained 24-epibrassinolide [(22*R*,23*R*,24*R*)-2α,3α,22,23-tetrahydroxy-24-methyl-7-oxa-7a-homo-5α-cholestan-6-one; (**6**)] directly from 24-epicastasterone on treatment with trifluoroperoxyacetic acid. The product **6** was obtained as fine white needles with m.p. 257–258 °C, with IR, NMR and analytical data identical with those in the literature[3,17].

Cell Culture

Mouse hybridoma cell line ME-750, producing an IgG monoclonal antibody, was cultured in DMEM/F12/RPMI 1640 (2:1:1) medium supplemented with amino acids and with the iron-rich protein-free growth-promoting mixture as described in detail previously[18,19]. Cell culture media and supplements were from Life Technologies. The media for cell starvation were prepared by diluting the above defined media with phosphate-buffered saline supplemented with the iron-rich growth-promoting mixture. The cultures were kept at 37 °C in a humidified atmosphere containing 5% CO_2.

Compound **6** was dissolved initially in dimethyl sulfoxide to 10 mmol l^{-1} concentration. Further dilutions were prepared in the culture medium.

Assays of the action of **6** were set up in a total volume of 6.0 ml in 25 cm^2 T-flasks in the semicontinuous culture mode. In semicontinuous cultures, a standard fraction of the culture volume was withdrawn in a stepwise mode once a day, and replaced by medium containing the respective concentration of **6** (ref.[19]). Viable and dead cells were counted in a hemocytometer using the Trypan Blue exclusion test. The concentration of monoclonal antibody (further abbreviated MAb) in the culture supernatant was determined by immunoturbidimetry in three parallels for each sample[20] .

The values of viable and dead cell concentrations, as well as the values of the monoclonal antibody (MAb) concentration were determined daily. Four to five values of cell concentrations and of MAb concentrations, obtained on individual days of culture, were averaged to obtain steady-state mean values.

Distribution of Cell Cycle Phases

For the determination of cell kinetics, the cells (5–10 × 10^6) were permeabilized and stained using DNA Prep kit (Beckman Coulter, Hialeah, U.S.A.) following the producer's protocol. After 30-min incubation, the samples were measured with a FACSCalibur flow cytometer (Beckton Dickinson, Franklin Lakes (NJ), U.S.A.), and the data were analysed by ModFit LT 3.1 DNA analysis software (Verity Software House, Inc., Topsham (ME), U.S.A.). Percentages of cells in G_0/G_1, S and G_2/M phases were evaluated.

Mitochondrial Membrane Potential

The flow cytometer was used to analyze cells, stained for mitochondrial potential using Rhodamine 123 (Sigma–Aldrich, Prague). The cells were diluted with the medium to a concentration of 5–6 × 10^5 cells ml^{-1}, stained with a final concentration of 2 μg ml^{-1} Rhodamine 123, and incubated at 37 °C for 10 min. The cells were centrifuged, resuspended in phosphate buffered saline, and stored on ice in the dark. The stained cell were analysed within 15–30 min at the excitation wavelength 488 nm and the emission wavelength 530 nm (ref.[21]). The mitochondrial potential was evaluated as mean fluorescence.

Intracellular Antibody

Intracellular level of MAb's was measured by flow cytometry. Cells (2–5 × 10^5) were permeabilized using FIX&PERM Cell Permeabilization Kit (Kaumberg, Austria) according to the manufacturer's recommendations, and stained by phycoerythrin-labelled Goat Anti-Mouse IgG polyclonal antibody $F(ab')_2$ fragment (Beckman Coulter, Nyon, Switzerland). The MAb content in individual cells was evaluated as mean fluorescence at 585 nm.

RESULTS

In the present experiments, we employed the methodical approach of semicontinuous culture, based on the chemostat mode of cell culture[22]. This mode of cell culture allows to suppress possible transient effects appearing upon administration of a single dose of the tested substance, and to obtain reliable sets of data for statistical evaluation from a steady-state culture.

Exploratory batch experiments carried out with concentrations of **6** in the range 10^{-12}–10^{-6} mol l^{-1} showed culture parameters significantly different from those of the control only at the lowest concentrations tested. Therefore, in the final semicontinuous culture experiments, a lower concentration range was selected, *i.e.* 10^{-16}–10^{-9} mol l^{-1}.

The control culture was carried out in a standard protein-free medium. In order to guarantee a steady excess of nutrients, the dilution rate was adjusted to 0.5 day^{-1}. The resulting viable cell density was approximately 1.9 × 10^6 cells ml^{-1}, and the culture viability was more than 90% (Table I). As expected, under these conditions, **6** did not induce any change in the viable cell density or viability. Similarly, no significant effect on the MAb

concentration in the medium was found. However, even under the conditions of an excess of nutrients, significant changes in the level of intracellular MAb, and in the distribution of cell cycle phases could be observed even at the lowest concentration of **6** (Table II). At all concentrations of **6** tested, the proportion of the G_0/G_1 phase was higher, and the proportion of the S phase was lower than in the control culture. An elevated mitochondrial membrane potential, and a lower value of the intracellular MAb concentration, relative to the control, were found at 1×10^{-15} mol l^{-1} and higher concentrations of **6**.

The task of culturing cells under a severe nutritional stress was fulfilled by suppressing the concentrations of all media components to 30%, and, moreover, by decreasing the dilution rate to 0.25 day^{-1}. Consequently, the steady-state viable cell density was only *ca* 0.3×10^6 cells ml^{-1}, and the culture viability between 62 and 68% (Table III). The anti-stress activity of **6** manifested itself at concentrations in the range 10^{-12}–10^{-13} mol l^{-1}. In this concentration range, the steady-state viable cell density was higher by more than 15% in comparison with the control experiment. The viability was

TABLE I
Semicontinuous cultures in standard medium at the dilution rate $D = 0.5$ day^{-1}. Cell culture parameters

Concentration of **6** mol l^{-1}	Viable cell density[a]	Viable cell density/total cell density	MAb in medium[a]	MAb intracellular[a]	Mitochondrial membrane potential[a]
0 (Control)	1.00 ± 0.04	0.93	1.00 ± 0.08	1.00 ± 0.03	1.00 ± 0.04
1×10^{-16}	1.00 ± 0.04	0.91	0.86 ± 0.07	0.91 ± 0.04	1.01 ± 0.01
1×10^{-15}	0.98 ± 0.01	0.90	0.90 ± 0.05	0.66 ± 0.08***	1.05 ± 0.01*
1×10^{-14}	1.00 ± 0.04	0.95	0.95 ± 0.09	0.80 ± 0.05***	1.07 ± 0.03*
1×10^{-13}	0.99 ± 0.04	0.92	1.06 ± 0.08	0.71 ± 0.09***	1.13 ± 0.02**
1×10^{-12}	0.99 ± 0.04	0.96	1.11 ± 0.05	0.79 ± 0.05***	1.06 ± 0.04*
1×10^{-11}	1.06 ± 0.10	0.93	0.97 ± 0.04	0.69 ± 0.03***	1.09 ± 0.02**
1×10^{-10}	1.01 ± 0.07	0.95	1.07 ± 0.12	0.84 ± 0.04***	1.09 ± 0.04**
1×10^{-9}	1.02 ± 0.05	0.92	0.98 ± 0.10	0.76 ± 0.10***	1.08 ± 0.03**

[a] Values relative to those in the control culture without compound **6**. Mean ± standard deviation values are given. Mean viable cell density in the control culture was (1890 ± 76) $\times 10^3$ cell ml^{-1}. Mean MAb concentration in the supernatant of the control culture was 16.9 ± 1.4 mg l^{-1}. Significantly different from control: * $p < 0.1$, ** $p < 0.05$, *** $p < 0.01$.

TABLE II
Semicontinuous cultures in standard medium at the dilution rate $D = 0.5\ day^{-1}$. Distribution of cell cycle phases

Concentration of **6** mol l^{-1}	G_0/G_1		S		G_2/M	
	%	relative to control	%	relative to control	%	relative to control
0 (Control)	49.1 ± 1.0	1.00	41.0 ± 2.1	1.00	9.9 ± 1.1	1.00
1×10^{-16}	50.5 ± 1.1*	1.03	38.5 ± 0.6*	0.94	11.0 ± 1.2*	1.11
1×10^{-15}	51.1 ± 0.8**	1.04	37.3 ± 0.4***	0.91	11.6 ± 0.9*	1.17
1×10^{-14}	53.0 ± 1.0***	1.08	33.6 ± 0.4***	0.82	13.4 ± 0.7***	1.35
1×10^{-13}	54.8 ± 1.3***	1.12	31.3 ± 0.9***	0.76	13.9 ± 0.7***	1.40
1×10^{-12}	56.9 ± 2.2***	1.16	31.5 ± 1.2***	0.77	11.6 ± 1.5	1.17
1×10^{-11}	53.9 ± 0.4***	1.10	34.4 ± 0.7***	0.84	11.7 ± 0.8*	1.18
1×10^{-10}	54.0 ± 0.8***	1.10	34.5 ± 1.8***	0.84	11.5 ± 1.6	1.16
1×10^{-9}	53.9 ± 0.9***	1.10	32.8 ± 1.9***	0.80	13.3 ± 1.2**	1.34

Mean ± standard deviation values are given. Significantly different from control: * $p < 0.1$, ** $p < 0.05$, *** $p < 0.01$.

TABLE III
Semicontinuous cultures in 30% medium at the dilution rate $D = 0.25\ day^{-1}$. Cell culture parameters

Concentration of **6** mol l^{-1}	Viable cell density[a]	Viable cell density/total cell density	MAb in medium[a]	MAb intracellular[a]	Mitochondrial membrane potential[a]
0 (Control)	1.00 ± 0.04	0.62 ± 0.06	1.00 ± 0.03	1.00 ± 0.03	1.00 ± 0.03
1×10^{-16}	1.00 ± 0.04	0.65 ± 0.04	1.00 ± 0.03	0.90 ± 0.05	1.01 ± 0.06
1×10^{-15}	1.04 ± 0.04	0.63 ± 0.04	0.98 ± 0.05	0.76 ± 0.05***	1.14 ± 0.07**
1×10^{-14}	0.98 ± 0.05	0.62 ± 0.05	0.97 ± 0.06	0.67 ± 0.05***	1.10 ± 0.06**
1×10^{-13}	1.16 ± 0.10**	0.68 ± 0.07	0.98 ± 0.09	0.69 ± 0.09***	1.07 ± 0.04*
1×10^{-12}	1.19 ± 0.10**	0.67 ± 0.05	0.93 ± 0.07	0.66 ± 0.05***	1.11 ± 0.04**
1×10^{-11}	1.01 ± 0.04	0.63 ± 0.04	0.96 ± 0.09	0.75 ± 0.03***	1.10 ± 0.06**
1×10^{-10}	1.00 ± 0.07	0.64 ± 0.05	0.92 ± 0.08	0.68 ± 0.04***	1.16 ± 0.05**
1×10^{-9}	1.04 ± 0.06	0.65 ± 0.04	0.95 ± 0.09	0.58 ± 0.10***	1.18 ± 0.08**

[a] Values relative to those in the control culture without compound **6**. Mean ± standard deviation values are given. Mean viable cell density in the control culture was $(330 \pm 16) \times 10^3$ cells ml^{-1}. Mean MAb concentration in the supernatant of the control culture was 9.7 ± 1.1 mg l^{-1}. Significantly different from control: * $p < 0.1$, ** $p < 0.05$, *** $p < 0.01$.

slightly higher, but the difference was not statistically significant. The increase in viable cell density was not accompanied by an increase in MAb concentrations in the medium. The effect of **6** on the level of intracellular MAb and on the mitochondrial membrane potential was analogous to that observed in the standard medium. The cultures in the diluted medium were characterized by a striking accumulation of cells in the S phase (Table IV). The general character of the shift of proportions between G_0/G_1 and S phases, which occurred even at the lowest concentration of **6**, was the same as in the standard medium, *i.e.*, increase of G_0/G_1 phase with a simultaneous decrease of S phase cells.

DISCUSSION

Recent studies on the mechanism of action of brassinosteroids in plants indicate that more than one molecular mechanism of brassinosteroid action exists: biosynthesis of enzymes and other physiological factors *via* an effect on genome expression, and an effect on membranes. These modes of action are obviously interconnected[6]. Brassinosteroids are known to act on the genome in the form of a hormone-receptor complex. The action of these substances results in genome activity regulations, and could be responsible for

TABLE IV
Semicontinuous cultures in 30% medium at the dilution rate $D = 0.25$ day^{-1}. Distribution of cell cycle phases

Concentration of **6** mol l^{-1}	G_0/G_1		S		G_2/M
	%	relative to control	%	relative to control	%[a]
0 (Control)	29.6 ± 1.8	1.00	70.2 ± 2.8	1.00	0.2 ± 0.2
1×10^{-16}	29.5 ± 1.2	1.00	69.4 ± 2.1	0.99	1.1 ± 0.3
1×10^{-15}	34.0 ± 1.3**	1.15	65.9 ± 2.1*	0.94	0.1 ± 0.2
1×10^{-14}	34.1 ± 1.2**	1.15	65.2 ± 2.9*	0.93	0.7 ± 0.5
1×10^{-13}	35.0 ± 1.5**	1.18	64.6 ± 2.5*	0.92	0.4 ± 0.3
1×10^{-12}	33.0 ± 1.4**	1.11	66.8 ± 1.5*	0.95	0.2 ± 0.2
1×10^{-11}	33.2 ± 1.5**	1.12	66.0 ± 2.2*	0.94	0.8 ± 0.5
1×10^{-10}	35.4 ± 2.1**	1.20	64.5 ± 2.8*	0.92	0.1 ± 0.2
1×10^{-9}	33.7 ± 1.6**	1.14	65.3 ± 3.4*	0.93	1.0 ± 0.6

[a] Due to very low percentage values and large standard deviations, additional statistical evaluation was omitted. Mean ± standard deviation values are given. Significantly different from control: * $p < 0.1$, ** $p < 0.05$.

the effects on transcription and DNA replication[23]. In addition, brassinosteroids are capable of influencing the electric properties of membranes, such as activation of the membrane-bound proton pump[6].

The present experiments were designed with the aim to compare the effect of **6** on a control "over-fed" culture with that on a culture carried out under a severe nutritional stress. Rather surprisingly, altered values of intracellular antibody, altered values of the mitochondrial membrane potential, and altered distribution of cell cycle phases were found even in the control culture. This finding leads to a suggestion that **6** may influence the ways of transcription and DNA replication irrespective of the nutritional status. A significant positive effect of **6** on the growth of the cultures under nutritional stress manifested itself in a zone of concentrations encompassing two orders of magnitude, namely 10^{-12} and 10^{-13} mol l^{-1} (see Table III). The lack of growth stimulation at concentrations 10^{-11} ml l^{-1} and higher may be explained by a complex action of **6** on several targets with different association constants. The cultures carried out in the diluted medium are characterized by an extremely high proportion of cells in the S phase, and by a relatively low viability value. These features obviously reflect the very slow and difficult process of the synthesis of cell components under an insufficient supply of substrates and energy sources.

The extremely low values of active concentrations of **6** indicate that the main mechanism of action of this compound is an alteration of gene expression, following the general scheme

6 + receptor → **6**-receptor complex → nuclear DNA → mRNA → protein (*e.g.* enzyme or factor in cell regulation).

The cell parameter alterations caused by the presence of **6** are in accord with the concept that brassinosteroids possess anti-stress activities. As documented by the increase in the mitochondrial membrane potential, **6** is able to support the activity of mitochondria, an organelle of crucial importance in the energetic metabolism. A more efficient conversion of substrates in energy available for cellular processes may represent one of the ways the cells overcome nutritional stress.

The suspension culture of mammalian cells in vitro is a system defined on a higher level than the plant models on which the growth-stimulating activity of brassinosteroids was revealed. The steady-state established in semicontinuous cultures allows us to precisely analyse the quantitative relationships between the number of cells and the number of molecules of the agent. Our finding of significant alterations of several culture parameters

at concentrations of **6** of the order of 10^{-15} mol l^{-1} indicates that the compound may be physiologically active even if the number of molecules of the agent in the system is of the same order of magnitude as the number of viable cells. The unusually low value of the active concentrations, ≈1 fmol l^{-1}, is conceivable under the assumption that multiple binding sites are involved in the interaction of **6** with the corresponding receptor. The final changes in the whole cell population obviously follow a cascade of events starting with an altered gene expression in a few cells that have been hit by the original molecular signal.

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