

environment of northwestern Europe in the early Holocene allows the possibility that Mesolithic hunters could have been responsible for the giant deer's final demise, although the terminal dates for *M. giganteus* pre-date the earliest human evidence in both the Isle of Man and Ireland<sup>14</sup>. Alternatively, it is possible that the Holocene vegetational zonation was unlike that of previous interglacials and so led to the demise of species adapted to a 'mosaic' environment<sup>12</sup>.

It is hard to generalize about causes of extinction as different factors may eliminate populations in different areas. Our new dates for *Megaloceros* add an important new species to the list of Holocene survivors from the Pleistocene world. For the woolly mammoth *Mammuthus primigenius*, the species most extensively dated, recent finds on Wrangel Island in the Siberian Arctic indicated an unexpected survival into the Holocene<sup>15</sup>. It is possible that we are witnessing a pattern of terminal survival of Pleistocene megafauna on islands at the margins of continents.

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Cell signalling

Control of free calcium in plant cell nuclei

Free calcium ions stimulate a huge variety of processes inside the cell<sup>1</sup>, eliciting specific responses that depend on their spatio-temporal concentrations<sup>2,3</sup>. Here we investigate how these changes in calcium concentration are triggered in the cytosol and nucleus of plant cells, and find that they are independently controlled in the two compartments. Our results indicate that in plants some processes in the nucleus may be executed in response to an autonomously regulated nuclear calcium signal, although it is not clear whether this happens in animal cells as well<sup>4,5</sup>.

To compare nuclear and cytosolic calcium signals in plant cells, we used protoplasts from transgenic tobacco plants that constitutively express the calcium reporter aequorin either in the cytosol<sup>6</sup> or in the nucleus<sup>7</sup>. The agent mastoparan<sup>8</sup> stimulated large and transient increases in free calcium in both the cytosol and nucleus in a dose-dependent manner, with the maximum increase in free-calcium concentration being in the micromolar ( $\mu\text{M}$ ) range for both compartments.

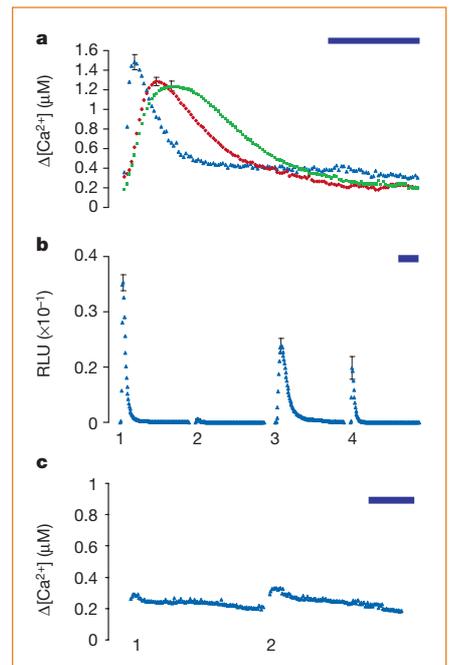
We found, however, that the cytosol and nucleus were differentially sensitive to mastoparan, with half-maximal (in terms of peak height) increases in free calcium in the cytosol and nucleus being elicited by 1  $\mu\text{M}$

and 5  $\mu\text{M}$  mastoparan, respectively (results not shown). The nuclear compartment consistently responded later than the cytosol, regardless of mastoparan concentration (the delay ranged from  $13 \pm 2.5$  s in the presence of 0.5  $\mu\text{M}$  mastoparan (not shown) to  $4 \pm 0.5$  s with 5  $\mu\text{M}$  mastoparan (Fig. 1a)).

We reasoned that the different response of the two cellular compartments to mastoparan might be due to a difference in the intracellular movement either of calcium itself or of a regulatory compound(s) from the cytosol to the nucleus, or to a specific and independent response by the nucleus itself. To test whether the nucleus could mount an autonomous calcium response to mastoparan, we compared the response to mastoparan of intact protoplasts with that of protoplasts that had been lysed under mild hypotonic-shock conditions<sup>9</sup> so that their nuclei were exposed.

As expected, 5  $\mu\text{M}$  mastoparan elicited an increase in free calcium in intact protoplasts expressing aequorin in either the cytosol or the nucleus (Fig. 1b, traces 1 and 3). Only a residual response (1–3%, relative to intact protoplasts) to mastoparan was recorded for the lysate prepared from protoplasts expressing aequorin in the cytosol (Fig. 1b, trace 2). In contrast, nuclear aequorin could still respond to mastoparan, with the size of the response being 70% of that by intact protoplasts (Fig. 1b, trace 4).

Under these conditions, addition of 1–10 mM calcium to the lysed nuclear



**Figure 1** Changes in free calcium concentration in the cytosol and nucleus of tobacco protoplasts. Protoplasts expressing the calcium reporter aequorin in the cytosol or in the nucleus are referred to as 'cyt' or 'nuc', respectively. **a**, Time course of changes in free calcium concentration in intact cyt (blue triangles), intact nuc (green squares) and lysed nuc (red diamonds) protoplasts induced in response to 5  $\mu\text{M}$  mastoparan. **b**, Responses of intact cyt (trace 1), lysed cyt (trace 2) and of intact nuc (trace 3) and lysed nuc (trace 4) protoplasts to 5  $\mu\text{M}$  mastoparan. RLU, relative light unit. **c**, Effect on nuclear  $\text{Ca}^{2+}$  concentration after addition of 1 mM (trace 1) or 10 mM (trace 2)  $\text{CaCl}_2$  to the incubation medium containing lysed nuc protoplasts. Scale bars, 10 s.

preparation had no effect on the existing concentration of free calcium (Fig. 1c), suggesting that the nuclear compartment is not passively permeable to calcium, at least in the form of isolated nuclei. The calcium response to mastoparan recorded from lysed protoplasts expressing aequorin in the nucleus was always earlier than that in the corresponding intact protoplasts in the presence of either 1  $\mu\text{M}$  (not shown) or 5  $\mu\text{M}$  mastoparan (Fig. 1a).

Taken together, our results suggest that nuclei from plant cells are capable of generating their own calcium signals independently of changes in calcium ion concentration in the cytosol.

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Cell biology

Risky immortalization by telomerase

Senescence naturally limits the proliferation of mammalian cells in culture, possibly by shortening the telomere regions at the ends of chromosomes during cell division<sup>1,2</sup>. In support of this idea, introducing TERT, the catalytic subunit of telomerase — the enzyme that maintains chromosome ends — into certain cell types can extend their lifespan and potentially immortalize them<sup>3,4</sup>. It has been proposed that treatment with exogenous TERT might be useful for cell-based therapies by allowing indefinite expansion of normal human cells without damaging their genomes<sup>5,6</sup>. But we show here that TERT-driven cell proliferation is not genoprotective because it is associated with activation of the *c-myc* oncogene.

Human mammary epithelial cell (HMEC) cultures normally stop dividing at 55–60 population doublings (PDs). We infected these cells with a human(h)TERT retrovirus at PD40 and maintained them until PD250 (ref. 4), then tested whether telomerase activity was essential for this immortalized phenotype by excising the hTERT retrovirus at PD150 using Cre recombinase<sup>7</sup>. The resulting HMEC–Cre cells were maintained for at least another 20 population doublings and we saw no decline in growth rates in either pooled cells or individual clones. We used Southern-ern blots of genomic DNA to confirm that TERT had been removed (data not shown). To our surprise, telomerase activity remained high compared with the control parental culture, which had undetectable activity (Fig. 1a).

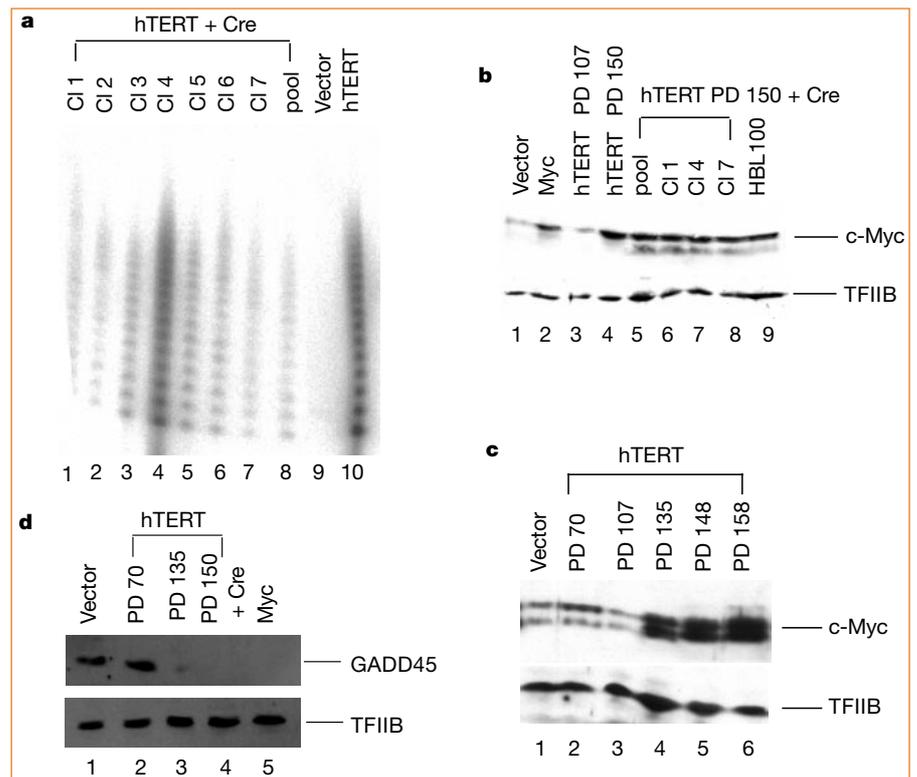
Ectopic expression of *c-myc* activates telomerase in HMECs<sup>4</sup>, and hTERT is a direct transcriptional target of *c-Myc*<sup>8</sup>. To determine whether activation of *c-myc* was

responsible for the telomerase activity found in our HMEC–Cre cultures, we measured the amount of Myc protein and found a two- to threefold increase in *c-Myc* compared with vector control cells (Fig. 1b). This is comparable to the amounts found in a culture immortalized by a *myc*-encoding retrovirus and in a breast-cancer cell line, HBL100 (Fig. 1b). The excision process did not itself cause this increase in *c-Myc*, as expression of *c-myc* was high before excision and also in clone 4, which still retained TERT (Fig. 1b).

We examined *c-myc* expression in HMEC–hTERT at different population doublings to determine when it was up-regulated and found that it increased between 107 and 135 population doublings (Fig. 1c). Also, GADD45 protein, whose expression is repressed by Myc, decreased markedly in HMEC–hTERT at PD135 and in HMEC–Cre at PD150 compared with vector control cells and HMEC–hTERT at PD70 (Fig. 1d). These results indicate that,

under standard culture conditions, extension of lifespan by telomerase selects for *c-myc* overexpression in HMECs.

Activation of the *c-myc* oncogene by overexpression, gene amplification, translocation and possibly mutation occurs in a wide variety of tumour types<sup>9</sup>. We have shown that, although telomerase activation extends the lifespan of HMECs, it is also associated with overexpression of *c-myc* and so is not indefinitely genoprotective (even though the chromosome number in such cells is normal; data not shown). Paradoxically, the extension of lifespan that is conferred by TERT causes *c-myc* activation, and this immortalizes cells, in part by activating TERT expression. Furthermore, in HMEC cultures, TERT expression has little, if any, immortalizing potential until the *p16* tumour-suppressor gene has been inactivated<sup>10</sup>. These findings indicate that the use of hTERT for expansion of normal human cells for therapeutic purposes must be approached with caution.



**Figure 1** *c-myc* activity is increased in immortalized HMEC–hTERT cells. HMEC 184 spiral K cells were provided by M. Stampfer. All HMEC-derived cultures were maintained in complete mammary epithelium growth medium (MEGM, Clonetics) under standard conditions<sup>11</sup>. **a**, Telomerase activity in HMEC–Cre cells. We infected HMEC–hTERT cells at PD150 with retroviruses that direct the expression of Cre recombinase, and generated a pool (lane 8) and seven clones (Cl; lanes 1–7). The pool and individual clones, except clone 4, lost the hTERT retroviral cassette. After maintaining cultures for a further 20 population doublings (PDs), we prepared cell lysates for TRAP assays<sup>12</sup>. Lane 9, vector control cells at PD40; lane 10, non-excised HMEC–hTERT cells at PD150. Each lane corresponds to 10,000 cells. Results were similar in clones obtained after a second round of subcloning of Cre clone 6. **b**, Immunoblot using rabbit polyclonal anti-*c-Myc* antibody (N-262, Santa Cruz) showing *c-Myc* in cell lysates from vector-infected HMECs (lane 1), cells immortalized by ectopic expression of *c-Myc* (lane 2), hTERT-expressing cells at PD107 (lane 3) and PD150 (lane 4), Cre-infected HMEC–hTERT pool (lane 5) and clones 1, 4 and 7 (lanes 6–8), and tumour-cell line HBL100 (lane 9). **c**, Immunoblot showing the amount of *c-Myc* in cell lysates from vector control cells (lane 1) and HMEC–hTERT cells at different PDs (lanes 2–6). **d**, Immunoblot using rabbit polyclonal GADD45 antibody (H-165, Santa Cruz), showing GADD45 in cell lysates from vector control cells (lane 1), hTERT-expressing cells at PD70 (lane 2) and PD135 (lane 3), Cre-infected HMEC–hTERT at PD150 (lane 4) and cells immortalized by ectopic expression of *c-Myc* (lane 5). In **b–d**, TFIIB protein was used to normalize loading.