locus. In agreement, the wing phenotypes (thickened veins and wing-edge loss) of $N^{nd\text{-}3}$ mutants are completely suppressed by *pk-sple*^{13} (data not shown). The inhibitory effect of Sple provides a second mechanism for polarizing Notch signalling in R3/R4 that could be coordinated by Fz/Dsh (Fig. 4d).

In conclusion, Notch signalling is activated in R3/R4 in response to Fz/Dsh and we propose that Fz/Dsh sets up an initial bias in Notch activity between R3 and R4 by promoting Dl activity and inhibiting Notch via Sple in a coordinated manner. Feedback in the Notch pathway amplifies this bias so that even a subtle variation in the amount of signal received by Fz in the equatorial (pre-R3) and polar (pre-R4) cells in each ommatidium would generate distinct cell fates. This explains how a signal from the equator could be interpreted by the whole field of ommatidia (Fig. 4e) and is likely to be of widespread significance in the development of polarized structures within planar epithelia. The asymmetrical expression of Notch-pathway genes detected in feather primordia is consistent with this model[24]. Furthermore, this mechanism for the coordinated regulation of Notch signalling can also explain how neural precursors develop at specific positions within competent proneural fields. □

Methods

Fly strains. Alleles used were: N^{ts1}, $N^{nd\text{-}3}$, *dsh*^{1}, fz^{R54}, Dl^{rf}, Dl^{6B}, *sple*^{1} and *pk-sple*^{13}. For analysis of *E(spl)* a combination between $Df(3R)NF1^{P1}$ (removing *NF1*, *E(spl)mδ* and *E(spl)mγ* promoter[25]; M.T.D.C. and S.J.B., unpublished data) and $Df(3R)E(spl)^{grob32.2}$ (removing all *E(spl)* genes and *NF1*) was used. Rescue of *NF1* activity[25] did not modify the eye phenotypes and no defects were observed in $NF1^{P2}/Df(3R)NF1^{P1}$, which eliminates *NF1* only[25]. For mis-expression studies we used heat-shock-inducible, intracellular Notch (*hs-N*^{icd}; ref. 15), a transmembrane-activated Notch derivative driven by *sevenless* enhancer (sev^{E}-$N^{\Delta ecd}$; ref. 17), and the Gal4/UAS-targeted expression system[26]. UAS lines were: *UAS-N*^{icd} (gift of M. Haenlin), *UAS-N*^{dn} (ECN, containing Notch extracellular and transmembrane domains[16]), *UAS-dsh*[19], *UAS-E(spl)mδ* and *UAS-E(spl)mβ*. These were combined with *sev-Gal4* (expressed in R3, R4, R7, mystery and cone cells) and/or *spalt-Gal4* (expressed in R3, R4 and cone cells).

For N^{ts} experiments, larvae were incubated at 30 °C for 6 h, returned to 25 °C for 10 h or until eclosion. N^{icd} expression was induced in *hs-N*^{icd} larvae by 2 h at 37 °C.

mδ0.5 transgenic lines. A 487-bp fragment from the 1.9-kb genomic *Hind*III fragment upstream of *E(spl)mδ* was amplified using the primers GATCTAGATGCCATCAGATGTCAGC and CTACTAGTCTTTTGGCGCACAGTCAC, digested with *Spe*I (filled in) and *Xba*I, and ligated into *Asp*718 (filled in) and *Xba*I sites of HZ50PL. Transgenic lines were established by injection in *cn*;*ry* flies using standard procedures and all lines gave identical patterns of expression.

Immunofluorescence. the following antibodies were used: rabbit anti-β-galactosidase (Cappell), rabbit anti-Bar[27], guinea-pig anti-Coracle[28], rat anti-Elav (Developmental Studies Hybridoma Bank), rat anti-Spalt (a gift of R. Barrio) and mouse monoclonal antibodies against β-galactosidase (Promega), E(spl) proteins[29], Rough[30] and Delta[20].

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Cyclosporine induces cancer progression by a cell-autonomous mechanism

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Malignancy is a common and dreaded complication following organ transplantation[1–4]. The high incidence of neoplasm and its aggressive progression, which are associated with immunosuppressive therapy, are thought to be due to the resulting impairment of the organ recipient's immune-surveillance system[5–9]. Here we report a mechanism for the heightened malignancy that is independent of host immunity. We show that cyclosporine (cyclosporin A), an immunosuppressant that has had a major impact on improving patient outcome following organ transplantation[4,5], induces phenotypic changes, including invasiveness of non-transformed cells, by a cell-autonomous mechanism.

Our studies show that cyclosporine treatment of adenocarcinoma cells results in striking morphological alterations, including membrane ruffling and numerous pseudopodial protrusions, increased cell motility, and anchorage-independent (invasive) growth. These changes are prevented by treatment with monoclonal antibodies directed at transforming growth factor-β (TGF-β). *In vivo*, cyclosporine enhances tumour growth in immunodeficient SCID–beige mice; anti-TGF-β monoclonal antibodies but not control antibodies prevent the cyclosporine-induced increase in the number of metastases. Our findings suggest that immunosuppressants like cyclosporine can promote cancer progression by a direct cellular effect that is independent of its effect on the host's immune cells, and that cyclosporine-induced TGF-β production is involved in this.

We explored an alternative and autonomous cellular mechanism

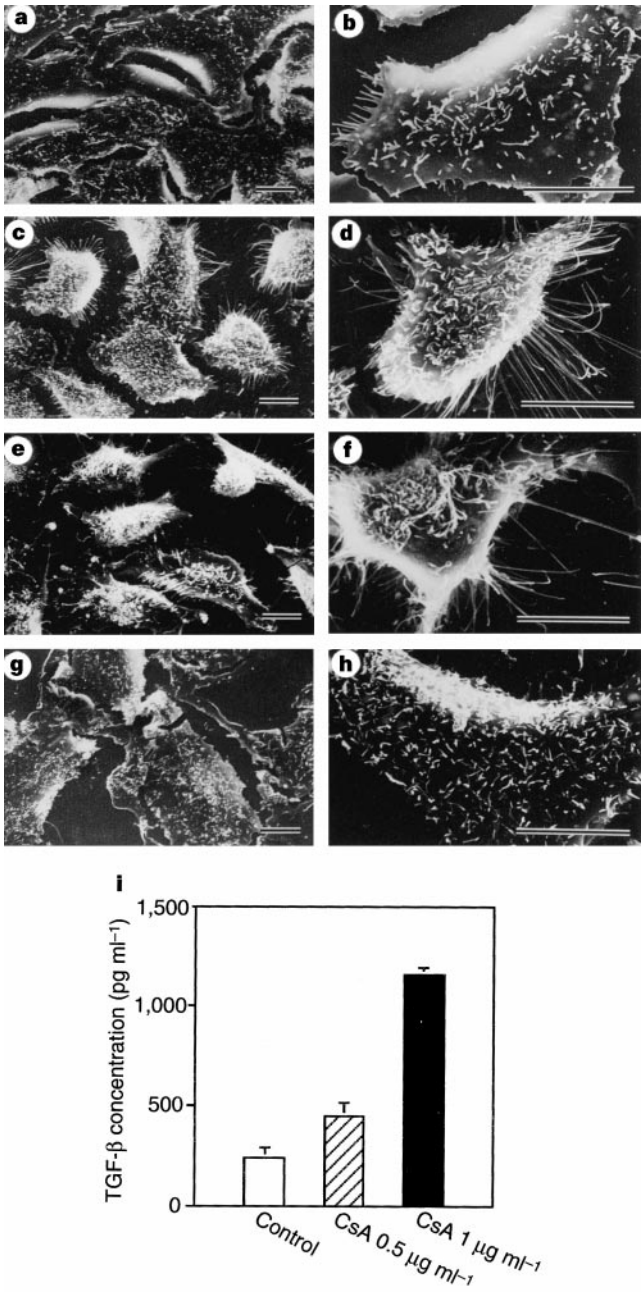

Figure 1 Cyclosporine induces A-549 cells to acquire an invasive phenotype. Scanning electron microscopic photographs of A-549 cells grown on glass coverslips and incubated for 72 h with: nothing (control cells, **a**, **b**); 1 μg ml^{-1} cyclosporine (**c**, **d**); 2 ng ml^{-1} recombinant TGF-β_1 protein (**e**, **f**); 1 μg ml^{-1} cyclosporine plus 30 μg ml^{-1} anti-TGF-β (**g**, **h**). Note that cyclosporine-conditioned cells, in a similar fashion to TGF-β_1-treated cells, display membrane ruffling and acquire exploratory pseudopodia, and that anti-TGF-β antibodies prevent these cyclosporine-induced morphological alterations. Scale bars, 10 μm. **i**, TGF-β concentrations (mean ± s.d.) in supernatants obtained from untreated or cyclosporine-treated A-549 cells. The cells were incubated for 72 h, in the absence or presence of 0.5 or 1.0 μg ml^{-1} cyclosporine, and a sandwich ELISA assay[11] was used to quantify TGF-β levels.

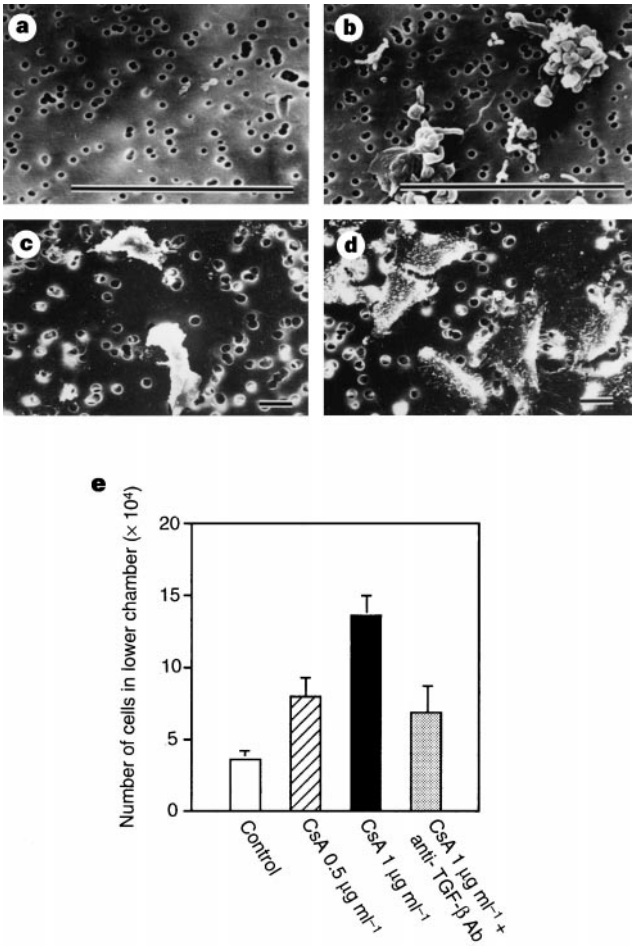

Figure 2 Cyclosporine stimulates the motility of A-549 cells. A-549 cells were grown for 72 h on 10-mm round polycarbonate membrane filters with 0.4 μm (**a**, **b**) or 3 μm (**c**, **d**) pore size in the absence (**a**, **c**) or presence (**b**, **d**) of 1 μg ml^{-1} cyclosporine. The filters were removed from the culture dishes and the bottom surfaces were examined by scanning electron microscopy. Note that the pseudopodia of cyclosporine-treated cells grown on 0.4-μm pore size filters protrude through the pores to the bottom surface (**b**) whereas cyclosporine-treated cells grown on 3-μm pore filters migrate through the pores onto the bottom surface (**d**). Scale bars, 10 μm. **e**, Cyclosporine promotes migration of A-549 cells. A-549 cells were placed in the upper chamber of 12-well transwells (8 μm pore) at a density of 10^5 cells, and were incubated alone or with 0.5 or 1.0 μg ml^{-1} cyclosporine or with 1.0 μg ml^{-1} cyclosporine plus 30 μg ml^{-1} anti-TGF-β antibodies (Ab). The cells that migrated into the lower chamber through the 8-μm pores of the polycarbonate membrane filters were counted after the cells had been dissociated by trypsinization. Results (mean ± s.e.m.) are of three experiments carried out with duplicate samples.

for immunosuppressant-associated tumour progression. We tested the hypothesis that cyclosporine, independently of any effects on the host immune system, would programme non-invasive cells to acquire an invasive phenotype. The experimental basis for our hypothesis was our demonstration that cyclosporine promotes the transcription and functional expression of the TGF-β_1 gene[10,11] and the observation by others that TGF-β can promote tumour-cell invasion and metastasis[12–14].

A non-transformed human pulmonary adenocarcinoma (A-549) cell line[14] that is not invasive *in vitro* was used as the indicator cell to test the hypothesis that cyclosporine can induce an invasive phenotype. A-549 cells express functional receptors for TGF-β, and their growth and function are regulated by TGF-β (refs 11, 14). We carried out these experiments *ex vivo* to avoid any confounding effects of cyclosporine-associated inhibition of *in vivo* immune surveillance mechanisms.

Figure 1 shows the striking morphological changes observed following cyclosporine treatment of A-549 cells. Scanning electron microscopic examination revealed that untreated A-549 cells display a cuboidal epithelial and non-invasive phenotype (Fig. 1a, b), whereas cyclosporine-treated cells show phenotypic alterations that are characteristic of invasive cells, that is, marked membrane ruffling and the formation of numerous pseudopodia (Fig. 1c, d). Additional data (Fig. 1) support the hypothesis that the cyclosporine-induced acquisition of an invasive phenotype is due to TGF-β. First, cyclosporine stimulated TGF-β secretion by A-549 cells in a concentration-dependent manner (Fig. 1i); second, anti-TGF-β monoclonal antibodies (1D11.16 $IgG)_1$[15], in contrast to control IgG_1 monoclonal antibodies, prevented the cyclosporine-induced morphological alterations (Fig. 1g, h); and third, recombinant TGF-β_1 induced morphological alterations in A-549 cells that were similar to those elicited by cyclosporine (Fig. 1e, f). Our finding that cyclosporine stimulates TGF-β_1 production in A-549 cells extends earlier observations that it induces T cells[10], CCL-64 mink lung epithelial cells[11] and renal cells[16] to hyperexpress TGF-β_1. The phenotypic changes elicited by cyclosporine were reversible; incubation of cyclosporine-treated A-549 cells in cyclosporine-free culture medium for 48 h resulted in the reversal of the invasive phenotype and a return to the original morphology (data not shown).

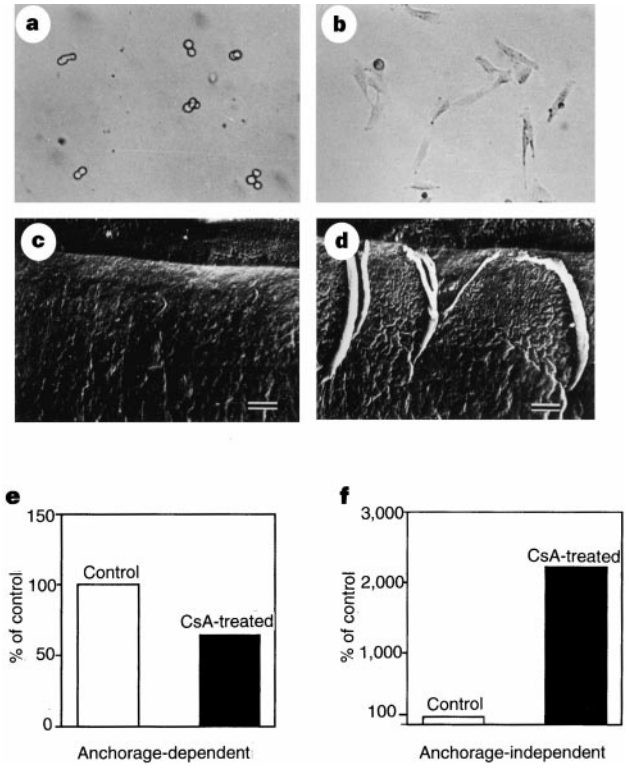

Figure 3 Anchorage-independent growth of cyclosporine-conditioned A-549 cells. Culture medium (3 ml) containing 10^4 cells and 1 μg ml^{-1} cyclosporine (**b**, **d**) or no cyclosporine (**a**, **c**) was loaded onto 5 ml of a 0.3% agarose layer containing MEM–10% FBS in 60-mm dishes. After 96 h incubation at 37 °C, cells grown on the surface of the agarose layer were examined with a phase-contrast microscope (**a**, **b**). For scanning electron microscopic observation (**c**, **d**), the soft gels were fixed and then vertical thin sections were made. These slices were processed for scanning electron microscopy as described in Methods. Twenty slices were made from each agarose plate, and representative ones are shown. Note that pseudopodia of cyclosporine-treated A-549 cells protrude deeply into the soft gel under anchorage-independent growth conditions. Scale bars, 10 μm. **e**, Cyclosporine-associated inhibition of anchorage-dependent proliferation of A-549 cells; **f**, cyclosporine-induced stimulation of anchorage-independent growth.

Cells capable of locomotion and invasiveness display exploratory pseudopodia[17,18]. Because cyclosporine induced numerous, long pseudopodia in A-549 cells, we investigated whether such cells acquired motility. To explore this, A-549 cells were seeded on polycarbonate membrane filters with three pore sizes (0.4, 3 and 8 μm) in the presence or absence of cyclosporine; and the bottom surfaces of the membrane filters were examined by scanning electron microscopy. Our results show that many cyclosporine-induced pseudopodia protrude through 0.4-μm pores onto the bottom surface (Fig. 2b), whereas only a few pseudopodia protrude in the control (Fig. 2a). When the cells were grown on 3-μm pore filters, many cyclosporine-treated cells and only few untreated cells, migrated through the pores to the bottom surface of the membrane filter (Fig. 2c, d).

To quantify the cell motility resulting from cyclosporine treatment, we used 8-μm pore filters in the migration assay. We found that the number of A-549 cells that migrated increased in proportion to the concentration of cyclosporine used to treat the cells, and that the increased cell motility was suppressed by anti-TGF-β antibodies (Fig. 2e), but not by the control IgG_1 antibodies. Thus, cyclosporine-induced alterations in both morphology (Fig. 1) and cell motility (Fig. 2) were dependent on cyclosporine-induced TGF-β production.

Anchorage-independent growth *in vitro* is considered a correlate of invasive tumour growth *in vivo*[19,20], and so we next examined whether cyclosporine treatment results in anchorage-independent growth. A-549 cells were plated on soft agarose gels and grown for 96 h (Fig. 3, see legend for details). Phase-contrast microscopic examination revealed that untreated A-549 cells retained their spherical shape and remained suspended in the culture medium, whereas cyclosporine-treated cells spread and proliferated strongly on the soft gel (Fig. 3a, b). Because it was difficult to determine by phase-contrast microscopy whether the pseudopodia extended along the surface of the agarose or penetrated deeper into the agarose layer, we made vertical thin sections of the soft gels and examined them by scanning electron microscopy to obtain side views. This strategy revealed that many fully grown pseudopodia of the cyclosporine-treated cells penetrated the agarose-gel layer and extended vertically into the gel plate (Fig. 3d). Also, these cells appeared to be supported by the extensively invaded pseudopodia, in contrast to the absence of pseudopodial extensions in the control A-549 cells (Fig. 3c).

Cyclosporine's effect on A-549 cells growth was contingent upon whether the culture conditions were anchorage-dependent or -independent. It inhibited the proliferation of A-549 cells under anchorage-dependent conditions but stimulated proliferation under anchorage-independent conditions (Fig. 3e, f).

We next examined whether cyclosporine induces morphological and functional alterations in other cell types, looking at murine renal cell adenocarcinoma (Renca) cells, mouse mammary gland epithelial (NMuMG) cells and mink lung epithelial (CCL-64) cells. We found that cyclosporine treatment produced phenotypic

alterations in these epithelial cells as well. A representative example (Fig. 4a, b) shows that cyclosporine-treated Renca cells, in a similar fashion to cyclosporine-treated A-549 cells, display an invasive phenotype.

We investigated whether cyclosporine would enhance the invasive and metastatic growth of tumour cells *in vivo* using Renca cells[21], and two other tumour cell lines, mouse-derived Lewis lung carcinoma cells[22] and human bladder transitional carcinoma cells[23], as the tumour inoculum. SCID–beige mice (mice homozygous for both *SCID* and *beige* mutations[24]), which are deficient in T cells, B cells and natural killer cells, were used as the host. The use of SCID–beige mice minimized the possibility that cyclosporine-induced depression of the host's immune system contributed to tumour progression.

Cyclosporine increased the number of murine renal carcinoma metastases in SCID–beige mice (Fig. 4c, d). Data from four separate experiments showed that the number of renal cell cancer pulmonary metastases was 241 ± 22 (mean ± s.e.m., $n = 21$) in the control SCID–beige mice compared with 338 ± 26 ($n = 18$) in the cyclosporine-treated mice ($P = 0.007$; *t*-test) (Table 1). Also, the number of pulmonary metastases resulting from inoculation of murine Lewis lung carcinoma cells was 11 ± 2 ($n = 9$ mice) in the control mice compared with 28 ± 4 ($n = 8$ mice) with cyclosporine treatment ($P = 0.003$), while the number of pulmonary metastases resulting from inoculation of human bladder transitional cancer cells was 63 ± 18 ($n = 9$ mice) in controls and 138 ± 21 ($n = 9$ mice) in cyclosporine-treated mice ($P = 0.01$) (Table 1).

We investigated the effect of anti-TGF-β antibodies (1D11.16 IgG_1)[15] on the cyclosporine-induced increase in the metastases to determine whether *in vivo* tumour progression by cyclosporine was dependent on TGF-β_1. Anti-TGF-β antibodies, but not control IgG_1 antibodies, prevented the cyclosporine-induced increase in metastases. The number of pulmonary metastases was 350 ± 22 (mean ± s.e.m., $n = 12$) in the control mice, 441 ± 20 ($n = 10$) in cyclosporine-treated mice, 284 ± 34 ($n = 8$) in mice treated with both cyclosporine and anti-TGF-β, and 490 ± 56 ($n = 4$) in mice treated with cyclosporine and control IgG_1 ($P = 0.0005$; one-way ANOVA). The reduction in the number of metastases found following the administration of anti-TGF-β antibodies to cyclosporine-treated mice was significant at $P < 0.01$ by ANOVA (Bonferoni *P*-value). In contrast, there was no significant difference between the number of metastases found in cyclosporine-treated mice and that found in mice treated with combined cyclosporine and control IgG_1 ($P > 0.05$). Our *in vitro* experiments show that the tumour cells are the sole source of TGF-β_1 (Figs 1, 2). Many cell types, in addition to tumour cells, might contribute to cyclosporine-induced TGF-β_1 hyperexpression *in vivo*.

The malignancy-promoting effects of immunosuppressive drugs are thought to result from drug-induced T-lymphocyte dysfunction and resultant immunosuppression. On the other hand, the production of TGF-β by tumours represents a potential mechanism by which they evade the host's immune system[25–28]. Our demonstration that cyclosporine-treated, non-transformed cells acquire invasiveness under *in vitro* conditions that allow no possible involvement of the host's immune system, and our *in vivo* data that cyclosporine promotes tumour growth in SCID–beige mice, suggest a cell-autonomous mechanism for cancer progression (Fig. 5). Specific therapeutic strategies that target pathways responsible for heightened invasiveness (such as TGF-β_1 regulation) are worth exploring and may be of value to people who are given allografts and to other individuals at increased risk of neoplasms. □

Table 1 Cyclosporine increases pulmonary metastases in SCID–beige mice

Tumour inoculum	Number of pulmonary metastases (mean ± s.e.m.)		
	Without CsA	With CsA	*P**
Murine Renca	241 ± 22 ($n = 21$)	338 ± 26 ($n = 18$)	0.007
Murine Lewis lung carcinoma (LLC)	11 ± 2 ($n = 9$)	28 ± 4 ($n = 8$)	0.003
Human bladder cancer (T24)	63 ± 18 ($n = 9$)	138 ± 21 ($n = 9$)	0.01

Tumour cells (1×10^6 or 5×10^5 in HBSS) were injected in the tail vein of SCID–beige mice. Cyclosporine (cyclosporin A; CsA; 20 mg per kg) was administered every other day from day −1 to the day of death. The mice were killed on day 19 (Renca), 16 (LLC) or 23 (T24), and the number of metastases was counted as described[30]. *n*, Number of mice in each group; *P* value derived with *t*-test.

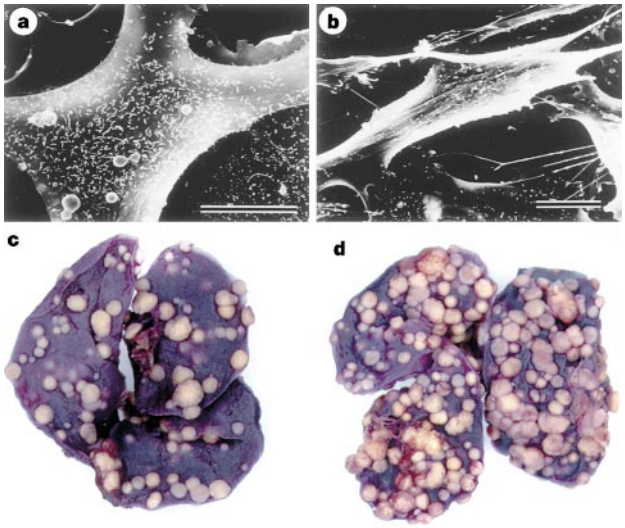

Figure 4 Cyclosporine induces renal cancer cells to acquire an invasive phenotype and promotes tumour growth *in vivo*. Scanning electron micrograph of murine renal adenocarcinoma cells incubated for 72 h in the absence (**a**) or presence of 1 μg ml^{-1} cyclosporine (**b**). Scale bars, 10 μm. Representative lungs, retrieved from untreated mice (**c**) and from cyclosporine-treated mice (**d**), are shown to illustrate the cyclosporine-associated increase in renal cell cancer pulmonary metastasis in SCID–beige mice.

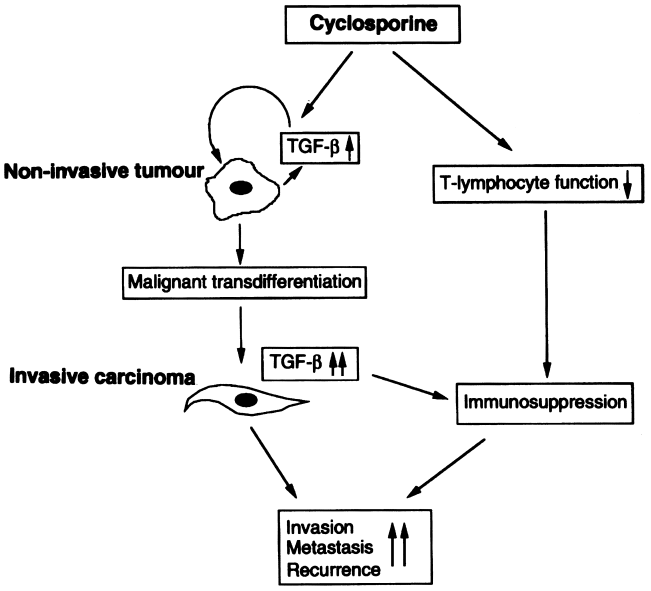

Figure 5 Potential mechanisms for cyclosporine-associated tumour progression. In this formulation, cyclosporine induced TGF-β production by tumour cells promotes cell invasiveness by a cell-autonomous mechanism that is independent of and/or complementary to cyclosporine's immunosuppressive effect on the host's immune system.

Methods

Cell line and culture. Human lung adenocarcinoma cells (A-549 cells; ATCC CCL 185; American Type Culture Collection, Rockville, MD), human bladder transitional carcinoma cells (ATCC HTB4, T24), mink lung epithelial cells (CCL-64; ATCC), mouse mammary gland epithelial cells (NMuMG; ATCC) and Lewis lung carcinoma cells (ATCC) were grown in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), at 37 °C in a 95% air–5% CO_2 atmosphere. Murine renal adenocarcinoma cells (Renca cells; a gift from R. H. Wiltrout, National Cancer Institute) were maintained by *in vivo* serial passages in syngeneic BALB/c mice, as described[21].

Scanning electron microscopy. Cells were seeded at a density of 10^5 on 12-mm round glass coverslips or 10-mm round polycarbonate membrane filters (0.4 or 3 μm pore size) in 12-well Transwells (Costar), and grown for 72 h in the presence or absence of cyclosporine. To assess the ability of TGF-β-specific antibody to inhibit cyclosporine-mediated effects, A-549 cells were incubated in the presence of both cyclosporine and monoclonal antibodies (Genzyme) that recognize TGF-β_1, -β_2 and -β_3. Cells were fixed with PBS, pH 7.4, containing 2.0% glutaraldehyde, and processed as previously described[29]. Samples were examined using a JEOL 25SIII electron microscope.

Quantification of TGF-β. TGF-β was quantified using a sandwich enzyme-linked immunosorbent assay (ELISA) method as previously described[11]. In brief, each well of multiwell ELISA assay plates was coated with anti-TGF-β_1 antibodies (1 μg ml^{-1}). The plates were incubated for 2 h at 37 °C after the addition of various amounts of TGF-β_1 in PBS or conditioned medium. After washing with PBS containing 0.2% Tween-20 (PBST), rabbit antiserum against TGF-β was added to each well. The plates were incubated at 37 °C for 1 h, the wells were washed with PBST, and then 100 μl of goat anti-rabbit IgG–alkaline phosphatase conjugates was added. Absorbance at 430 nm was measured using an ELISA assay reader. A-549 cells were cultured in serum-free medium to exclude contamination of cell-free supernatants by serum-derived TGF-β.

Cell proliferation assay. For assaying anchorage-dependent growth, A-549 cells were grown at a density of 2×10^4 cells per well in 12-well plates in the presence or absence of cyclosporine. After 96 h treatment, each well received 2 μCi of methyl-^{3}H-thymidine, and cells were incubated for an additional 4 h. They were washed twice with ice-cold PBS and fixed with methanol for 60 min. After washing, the fixed cells were lysed with 0.2 M NaOH and treated with cold 10% trichloroacetic acid (TCA) for 15–20 min on ice. The radioactivity, recovered as cold TCA-insoluble precipitates, was used for measuring relative cell proliferation by comparing the radioactivity between control and experiment. For an anchorage-independent cell growth, cells spread well on agarose gel were counted using a phase-contrast microscope.

***In vivo* tumour growth.** Murine renal cell adenocarcinoma cells (1×10^5 in Hank's balanced salt solution; HBSS), murine Lewis lung carcinoma cells (5×10^5 cells) or human bladder cancer cells (1×10^5 cells) were injected in the tail vein of 6-week-old male SCID–beige mice. Cyclosporine (20 mg per kg in 0.2 ml olive oil) was administered every other day starting from day −1, to day 19 or 23 after tumour inoculation. On day 19 or 23 after tumour inoculation, mice were killed and the number of pulmonary metastases was determined[30] following endotracheal insufflation of lungs with 15% India ink solution and bleaching the collected lungs in Fekete's solution. The effect of anti-TGF-β antibody and the control IgG_1 antibody on the cyclosporine-induced increase in the number of pulmonary metastases was determined by intraperitoneal administration of 200 μg of antibody, on a daily basis starting from day −1 to day 19 after tumour inoculation.

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RGD peptides induce apoptosis by direct caspase-3 activation

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Synthetic peptides containing the arginine–glycine–aspartate (RGD) motif have been used extensively as inhibitors of integrin–ligand interactions in studies of cell adhesion, migration, growth and differentiation[1–3], because the RGD motif is an integrin-recognition motif found in many ligands. Here we report