

Synthetic self-patterning and morphogenesis in mammalian cells: a proof-of-concept step towards synthetic tissue development

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Abstract: This study reports a proof-of-concept study as a step toward synthetic-biological morphogenesis of tissues. Events in normal animal development usually follow the sequence: patterning → differential gene expression → morphogenesis. A synthetic biological approach to development might follow a similar sequence, with each stage under the control of synthetic biological modules. The authors have constructed and published a synthetic module that drives self-organised patterning of mammalian cell populations into patches of different cell types. Here, as a proof of concept, they extend the self-patterning module with a morphogenetic effector that drives elective cell death in just one cell type. The result is a self-constructing pattern of two cell types, one of which can be selectively eliminated to leave remaining cells as a monolayer with a net-like structure. This simple device demonstrates and validates the idea of coupling synthetic biological morphogenetic effectors to synthetic biological patterning devices. It opens the path to engineering more sophisticated structures and, perhaps eventually, tissues.

1 Introduction

Synthetic biology constructs allow bio-engineers to endow living cells with properties that originate not in serendipitous evolution but in deliberate design [1–3]. Since the turn of the century, unicellular organisms have been engineered with synthetic biological systems to detect target molecules or light, to produce biofuels and pharmaceuticals, to perform logical computation and to hold and replicate encoded data (e.g. [4–10]). There is now increasing interest in applying synthetic biology approaches to multicellular systems, especially in higher plants and animals [11–14]. One important area of potential application is in tissue engineering. Classical tissue engineering operates by externally-managed construction of cell assemblies (e.g. by three-dimensional (3D) bio-printing, recent progress in which is reviewed in [15]) but there would be significant advantages to building new tissues from cells that organise themselves: such an approach would offer scalability, adaptability, maintainability and automatic error correction [16–18].

Our best model for understanding the self-organisation of tissues is natural embryonic development, in which cells have to organise themselves: there is no external source of information driving the detailed organisation in organisms such as mammals (some other organisms make use of light, gravity and flow to influence broad features, such as the direction of the root–shoot axis of plants). Most examples of natural development follow a sequence in which (i) a field of cells becomes patterned (e.g. by chemical gradients), (ii) cells in the different zones defined by the pattern express different sets of genes and (iii) these genes finally drive morphogenesis, which creates tissue anatomy [19]. Typically, during the development of a higher organism, this cycle repeats many times to add finer details as the organism grows. One method for constructing synthetic biological self-assembling structures would therefore be to follow this basic sequence: form a pattern → change gene expression → trigger morphogenesis.

Embryonic patterning de novo can take place by a variety of mechanisms, including reaction-diffusion, lateral inhibition and clock-and-wavefront mechanisms [20–25]. Existing patterns can also be made more elaborate, e.g. through interpretation of a monotonic concentration gradient as a series of stripes of different

cell states [26, 27]. We have recently published a simple synthetic biological system that uses a novel mechanism to drive spontaneous pattern formation, in a human cell line that does not normally show patterning activity. This synthetic system works through adhesion-mediated phase separation, to cause an initially random mix of cells to organise itself into cell patches with animal coat-like patterns [28].

Having constructed a synthetic patterning system, we are now adding a simple morphogenetic effector to transform a pattern only visible by fluorescent markers into physical form of the ‘tissue’. Of possible morphogenetic effectors (e.g. proliferation, apoptosis, cell scatter, sheet bending [11]), we chose apoptosis because it is expected to be rapid, cell-autonomous and the most likely to yield unambiguous results. If the green cells of a self-organised ‘animal coat’ pattern formed by green and red cells are induced to undergo apoptosis, elimination of the green cell patches would be expected to leave a reticular structure consisting of surviving red cells, and clear holes where green patches once were. We report that the idea works and produces the expected structure. The design is simple and is intended only as a proof-of-concept. Nevertheless, it could serve as a platform for the construction of more complex designs, by building up additional patterning systems and using more relevant morphogenetic effectors to generate complex 3D structures.

2 Results

The system for self-organised patterning, which we have already constructed and described [28], works by constrained, and therefore incomplete, phase separation. It uses a mix of two lines of engineered human TReX-293 cells, which naturally show low mutual adhesion. One line, ‘E-cells’, produces the cell adhesion molecule, E-cadherin, together with a Green Fluorescent Protein (GFP) reporter, when the cells are incubated in tetracycline. The other line, ‘P-cells’, produces the P-cadherin, together with a red mCherry reporter, under the same conditions. When mixed randomly and then given tetracycline, these cell populations attempt to separate to maximise contact with neighbours of the same type, because homophilic interactions between cadherins promote stronger adhesion than heterophilic interactions. This

creates alternating patches of cells of one type and the other (Fig. 1a, inset), in a statistically non-random pattern [28]. Having produced this pattern-generating system, we wished to implement a morphogenetic outcome from the pattern by inducing an apoptosis module in the E-cells, to generate a reticular sheet of P-cells with holes where the E-cells once were.

2.1 Construction of pattern-capable cells with an inducible apoptosis module

To confer inducible apoptosis on the E-cells, we used a *myrCasp8-ERT2* module developed as a tamoxifen-inducible, apoptosis-inducing caspase protein [29]. We cloned this *myrCasp8-ERT2* cassette into a pT-REx vector to add a second level of regulation on the apoptosis effector: upon tetracycline addition, the myristoylated and membrane-targeted caspase is strongly expressed, but remains inactive unless tamoxifen (4-Hydroxytamoxifen, 4-OHT) is added. The tamoxifen causes the caspase molecules to dimerise through their ERT2

tamoxifen-binding elements, and to enter the nucleus (Fig. 1a). Thus, we engineered cells with {tetracycline-AND-tamoxifen}-inducible apoptosis (Fig. 1b). We designated the modified E-cells as E^A-cells, the 'A' indicating the presence of the apoptosis module.

Cultured on their own without inducers for 54 h, E^A-cells formed a loose monolayer with no significant evidence of apoptosis (Fig. 2b). The E^A-cells did, however, grow a little more slowly than parental E-cells (Fig. 2a). E^A-cells, treated with tetracycline only, formed adhesive clusters (Fig. 2e), as would be expected from their expression of E-cadherin. Parental E-cells did not show such clusters despite E-cadherin expression, because of cell confluency (Fig. 2d). Tetracycline treatment of E^A-cells also induced a little amount of apoptosis, presumably due to leakiness of the tamoxifen switch (Fig. 2e). Treatment with tamoxifen alone produced cultures similar to those treated with no inducers (Fig. 2h). E^A-cells incubated in tetracycline for 48 h and in tetracycline and tamoxifen together for the final 6 h of the experiment showed a massive apoptotic response (Fig. 2k): the monolayer broke up and left highly condensed remains of dead or

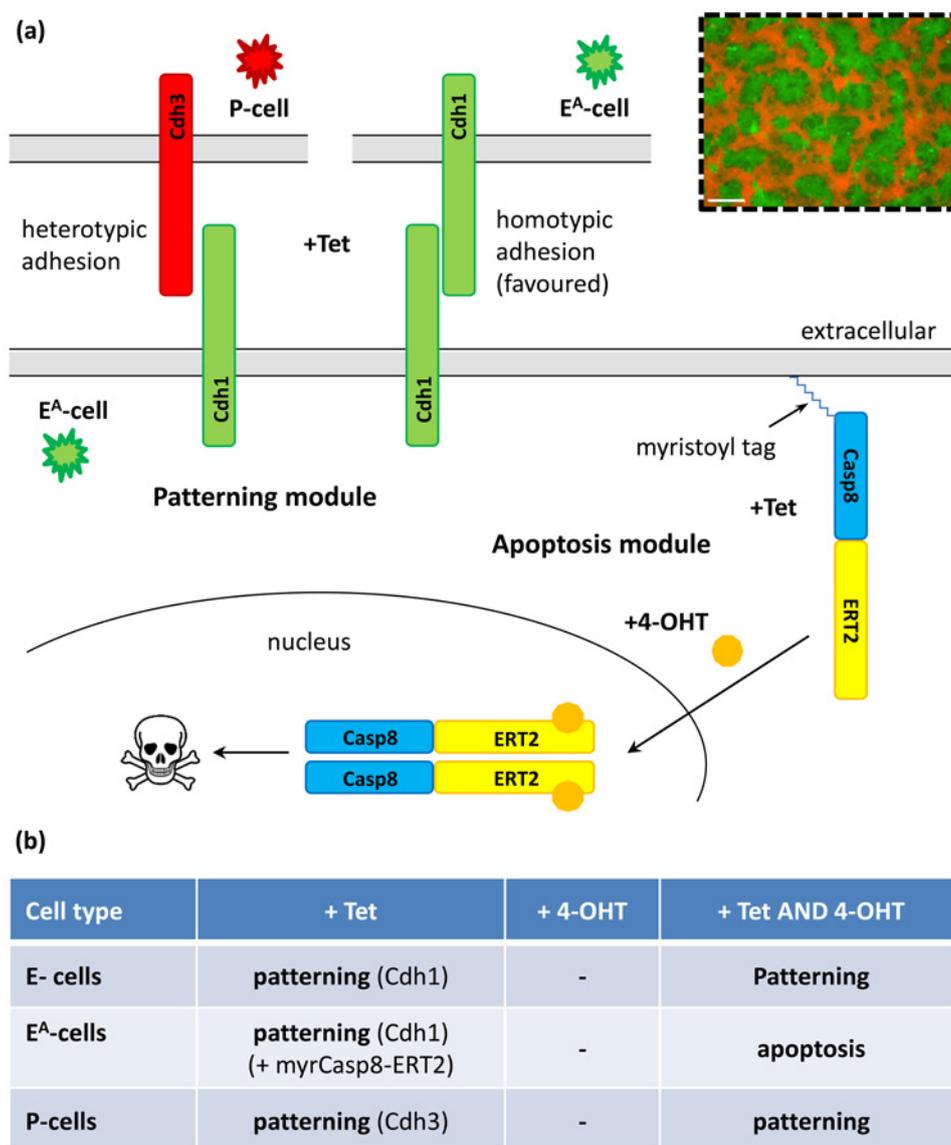


Fig. 1 Synthetic modules used in this study

a Schematic of the patterning and apoptosis modules. Patterning module: upon tetracycline (Tet) induction, cells express their respective cadherins which drive patterning through differential adhesion (insert, Cachat *et al.*, 2016 [28]). Apoptosis module: upon tetracycline induction E^A-cells also express Caspase-ERT2 fusion proteins targeted to membranes through a myristoyl tag. Only upon additional treatment with tamoxifen (4-OHT) do the Caspase-ERT2 proteins translocate to the nucleus and trigger apoptosis through dimerisation and caspase activation

b Cell behaviours induced by tetracycline and/or tamoxifen in the different cell types used in this study
Scale bar: 200 μ m

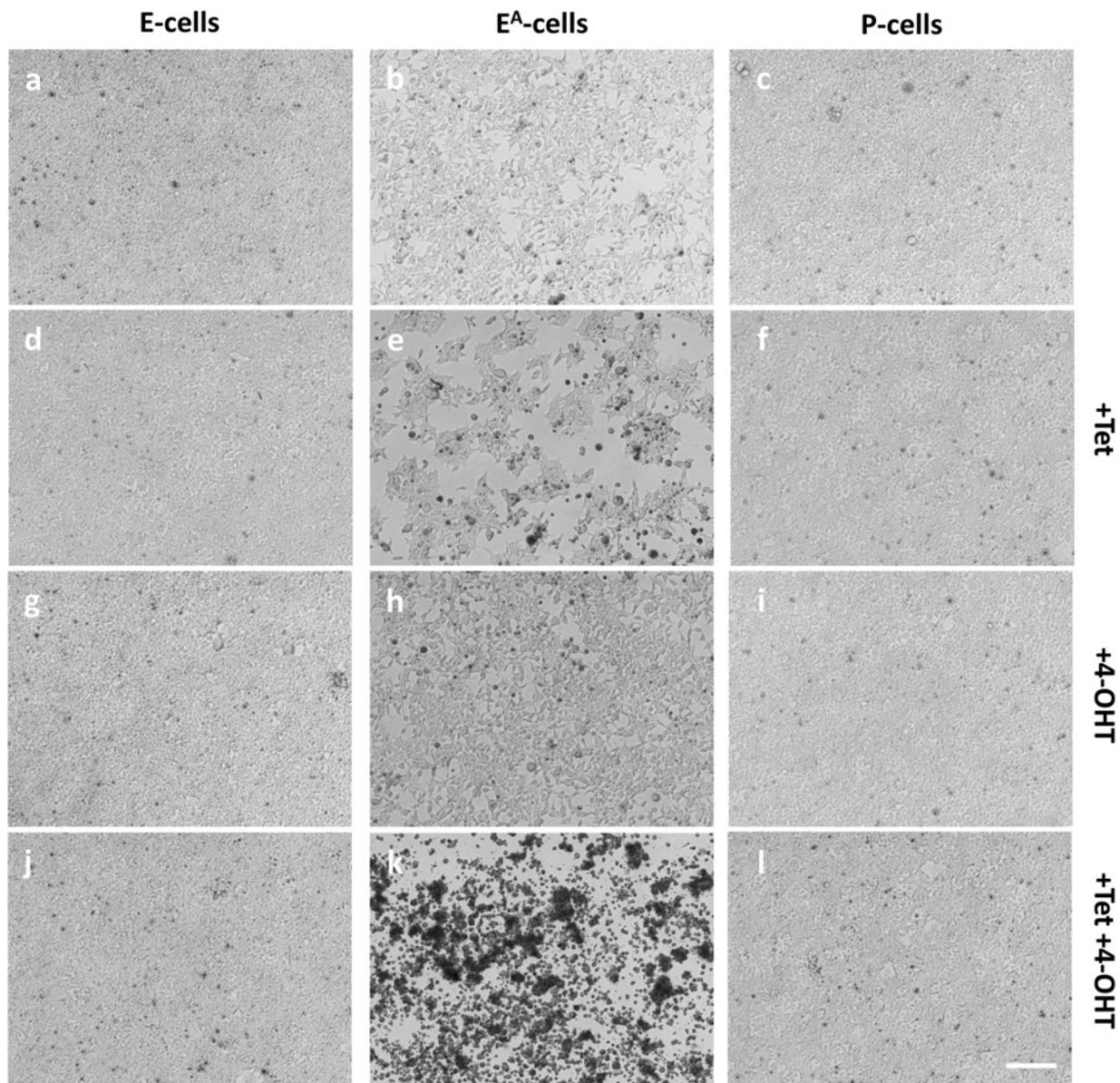


Fig. 2 Testing the tamoxifen-inducible apoptosis module in E^A -cells in the absence of patterning

a-c E-, E^A - and P-cells after 54 h growth in plain medium, showing normal cell survival

d-f Cells cultured for 54 h in tetracycline (Tet) only; E^A -cells have formed tetracycline-inducible adhesion clusters and a low amount of cell death

g-i Cells cultured for 54 h in tamoxifen (4-OHT) only; the cultures have remained healthy, showing that tamoxifen is not itself cytotoxic

j-l Cells treated for 48 h with tetracycline alone, then for a further 6 h with tetracycline and tamoxifen: the E^A -culture consists of dying cells and their remains

Scale bar: 200 μ m

dying cells and their debris. The plain E- or P-cells did not show any significant apoptosis in any combinations of tetracycline and/or tamoxifen.

When E-cells and P-cells' cadherin expression is induced with tetracycline and the cells are mixed, they spontaneously produce patches of green-fluorescing E-cells and red-fluorescing P-cells (Fig. 3*a*), as observed before [28]. When E^A -cells are used in place of E-cells, under tetracycline induction and in the absence of tamoxifen, the patterning proceeds normally (Figs. 3*b* and *b'*). The seemingly slight 'background' stain observed from the E^A -cells in the red channel is due to low levels of mCherry being produced by E^A -cells in addition to their production of GFP, because mCherry is a component of the myrCasp8-ERT2-IRES-mCherry apoptosis cassette under tetracycline induction. Nevertheless, the patterned conformations observed here, especially clear in the overlay images, indicates that the mere presence of the myristoylated caspase 8, not yet activated by tamoxifen, does not prevent patterning by phase separation.

2.2 Patterning followed by apoptosis to create a reticulum

To perform the proof-of-concept experiment of patterning followed by a morphogenetic event, we induced mixtures of E^A -cells and P-cells to undergo phase separation by treating them with tetracycline, then treated the patterned cultures with tamoxifen to induce selective apoptosis of E^A -cells. As a control, we performed the same treatment on plain E- and P-cells patterns, which formed patterns as normal (Fig. 4*a*). In the E^A /P-cell patterns, the E^A -cells showed evidence of apoptosis within 6 h of tamoxifen being applied, the cells rounding up. Rinsing the cultures with medium removed most of the E^A -cells to leave a reticular sheet of red-fluorescing P-cells permeated with holes where the E^A -cells once were (Figs. 4*b* and *b'*). A small number of E^A -cells remained, generally rounded up but still visible in the GFP channel. The character of the reticulum was similar to that of the P-cells in the E^A /P mixed pattern (Figs. 3*b* and *b'*), with one difference: the

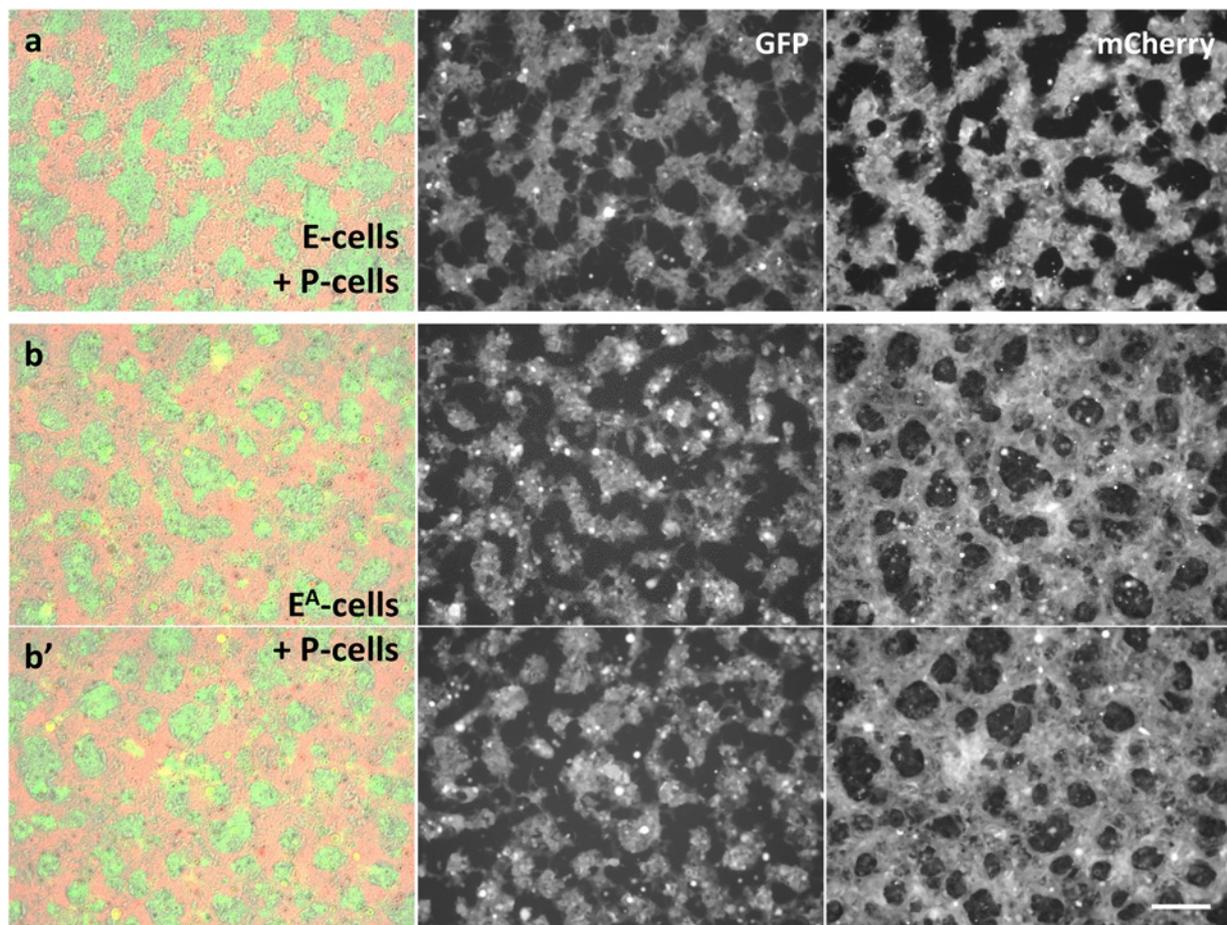


Fig. 3 Tetracycline-inducible patterning by mixtures of E^A - and P-cells

a shows patterning by plain E- and P-cells (i.e. the parental lines, not carrying the apoptosis module) 24 h after seeding under tetracycline induction: separation of green E-cells and red P-cells into different patches is apparent and has the same character we described in Cachat *et al.*, 2016 [28]

b, b' shows very similar patterning by a mixture of E^A - and plain P-cells under the same conditions, indicating that the presence of the uninduced apoptosis module does not affect patterning

Scale bar: 200 μ m

holes in the reticulum were smooth-edged, presumably because of surface tension.

The resulting reticulum is reproducible as shown in Fig. 4 (see original images and extra repeats in the dataset available from the Edinburgh DataShare repository [30]).

3 Conclusion

Overall, this simple study demonstrates the validity of the idea of coupling a synthetic biological patterning system with a morphogenetic effector to create physical shapes. Experimentally, this system follows a synthetic developmental script where (i) gene expression is induced (cadherins) and (ii) higher adhesion results in (iii) pattern formation. A second round of (iv) new gene expression (caspase), induces (v) cell death, which results in a new structure: the reticulum.

Each module of this synthetic cycle (patterning \rightarrow change in gene expression \rightarrow morphogenesis) can in theory be implemented in various orders and the cycle repeated numerous times to create increasingly complex structures. In this case, the simple nature of the apoptosis effector created a very simple shape (a reticulum), and one that will be strictly temporary: the ability of cells to repair holes in monolayers is well known and used as a common assay for cell vitality [31, 32]. The system was conceived as a demonstration only, and not to fulfil a practical purpose. Nevertheless, in principle more subtle effectors (causing cell bending or cell proliferation for example) might be used in future to create 3D shapes.

The approach outlined here is an alternative, and perhaps a complement, to methods of biological structure assembly that are inspired primarily by engineering (rather than embryology). One such interesting method is that of Todhunter *et al.* [13], who attached oligonucleotides to the surfaces of cells: cells could bind complementary oligonucleotides on either substrates or other cells, in order to build single- or multi-layered structures. Other methods that lie between the mechanical and the embryological include (i) seeding cells on microscope slides that were previously printed with cell-accepting or cell-repelling shapes [33], (ii) 3D printing of cell-laden substrates such as alginate [34] and (iii) production of cell-laden alginate tubes by dip-coating [35]. These methods, based on engineering principles, may prove to be of great practical use to tissue engineering. However, the embryology-inspired approach described here offers a use beyond the practical: it might in future be used to test theories of natural embryology [36], by constructing self-patterning-then-morphogenesis systems modelled on our current understanding of natural development, and so act as a powerful test of that understanding.

4 Methods

4.1 Constructs

The myrCasp8-ERT2 cassette was amplified from plasmid pCAG-MyrCasp8ER(T2)-bpA kindly donated by Ralf Kühn [29] and inserted in pDONR-221 kindly donated by Roure *et al.* [37], before shuttling into pTREx-DEST-IRES-mCherry [38] through

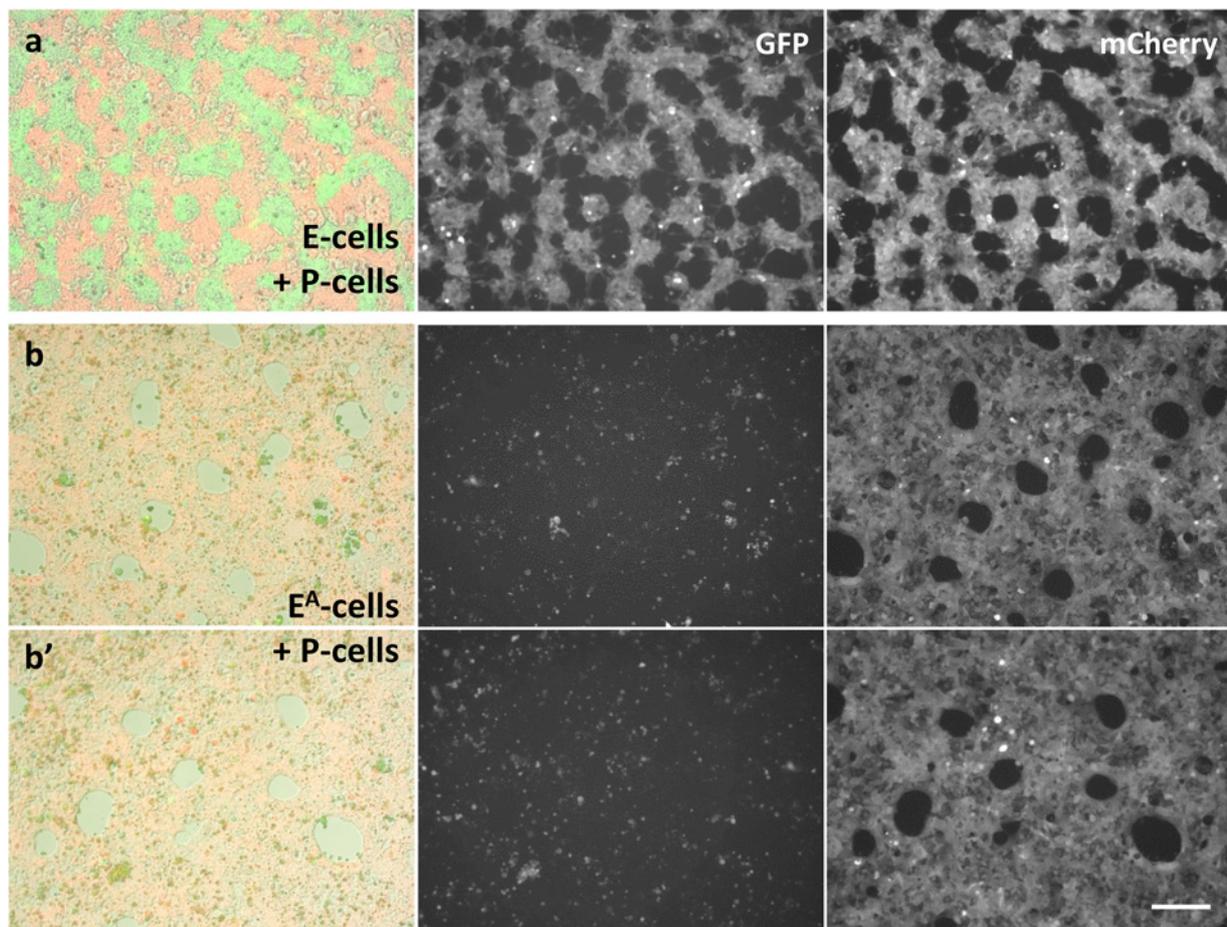


Fig. 4 Patterning followed by apoptosis to create a reticulum

a As a control, patterned mixtures of plain E- and P-cells (treated with tetracycline for 24 h, as in Fig. 3*a*), were treated with tamoxifen for a further 6 h with no effect on the pattern *b, b'* Under the same conditions, patterned mixtures of E^A- and plain P-cells (as in Fig. 3*b, b'*) form a reticulum: the green-fluorescing E^A-cells largely disappear, leaving holes where they once were, and leaving the culture as a reticulum of P-cells. All cultures were rinsed after tamoxifen treatment to remove floating dead cells
Scale bar: 200 μ m

Gateway® recombination according to manufacturer's instructions (Invitrogen), to create plasmid pTREx-myrCasp8-ERT2-IRES-mCherry.

4.2 Cell culture, transfections and clonal selection

E- and P-cells (Cachat *et al.* [28]) were maintained in DMEM (Gibco 41966) supplemented with 10% FBS (Biosera), 5 mg/mL blasticidin (Gibco) and 200 mg/mL G418 (Sigma), at 37°C and 5% CO₂. Cells were transfected with plasmid pTREx-myrCasp8-ERT2-IRES-mCherry and lipofectamine 2000 (Invitrogen) and maintained for two weeks under 800 mg/mL G418. Individual clones were isolated and tested for apoptosis under co-induction with 0.5 μ g/mL tetracycline (Sigma) and 1 μ M tamoxifen (4-OHT, Sigma).

4.3 Apoptosis, pattern formation and imaging

To induce apoptosis of E^A-cells, cells were pre-induced with 0.1 μ g/mL tetracycline for a total of 48 h before the addition of 1 μ M tamoxifen (4-OHT, Sigma) and results were recorded 6 h after. For pattern formation, cells were pre-induced with 0.1 μ g/mL tetracycline for 24 h before seeding in 24-well plates at equal ratio and grown for 24 h in 0.1 μ g/mL tetracycline. Selective apoptosis of E^A-cells was induced within 6 h after addition of tamoxifen. Images were acquired using a Zeiss AxioObserver D1 inverted fluorescence microscope with AxioCam MRm and 10 \times or 20 \times objectives. Filter excitation (Ex) and emission (Em) bandpass specifications were as follows (in nm): GFP (Ex: 470/40, Em: 525/50) and mCherry (Ex: 545/25, Em: 605/70). Fluorescence

contrast was enhanced across whole images for better rendering of patterns (with no impact on pattern features) and original images are available from the Edinburgh DataShare repository [30].

5 Data statement

All raw images and repeats are available from the Edinburgh DataShare repository [30].

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