Soft fibrin gels promote selection and growth of tumorigenic cells

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The identification of stem-cell-like cancer cells through conventional methods that depend on stem cell markers is often unreliable. We developed a mechanical method for selecting tumorigenic cells by culturing single cancer cells in fibrin matrices of ~100 Pa in stiffness. When cultured within these gels, primary human cancer cells or single cancer cells from mouse or human cancer cell lines grew within a few days into individual round colonies that resembled embryonic stem cell colonies. Subcutaneous or intravenous injection of 10 or 100 fibrin-cultured cells in syngeneic or severe combined immunodeficiency mice led to the formation of solid tumours at the site of injection or at the distant lung organ much more efficiently than control cancer cells selected using conventional surface marker methods or cultured on conventional rigid dishes or on soft gels. Remarkably, as few as ten such cells were able to survive and form tumours in the lungs of wild-type non-syngeneic mice.

The notion of ‘stem-cell-like cancer cells’ or tumorigenic cells has been based on the observation that only a very small fraction of cells from a tumour can seed and generate a tumour in a severe combined immunodeficiency (SCID) mouse1–3. These tumorigenic cells are often speculated to be the key players in the relapses after chemotherapy or surgery. However, the idea of stem-cell-like cancer cells is rather controversial1–5. A report shows that more than 25% of melanoma cells from human subjects, not just a small fraction, with no expression of a stem cell marker cluster of differentiation 133 (CD133), can seed and generate a tumour in a non-obese diabetic (NOD)-SCID interleukin-2 receptor gamma chain null (IL2ry−/−) mouse6, casting doubts on whether stem-cell-like cancer cells truly exist or whether they are biologically relevant to cancer. In contrast, a tiny heterogeneous subpopulation of cells from human colon cancer patients exhibits non-stochastic self-renewing capabilities and tumorigenicity, although these behaviours are also not correlated with stem cell markers7. Other reports show, however, that non-stem-like cancer cells seem to spontaneously and stochastically turn into stem-like cancer cells de novo8,9, suggesting that there is a bi-directional conversion between stem and non-stem states, further complicating the concept of stem-cell-like cancer cells. As a result, existing methods based on the correlative stem-cell-like cancer cell markers are unreliable. Therefore, new approaches are highly desirable for the study of tumorigenic cells.

We have recently demonstrated that mechanical forces can regulate mouse embryonic stem cell (ESC) differentiation and self-renewal independent of soluble factors and that mouse ESCs are approximately ten times softer than their differentiated counterpart cells10. Soft substrates maintain self-renewal of ESCs but stiff substrates promote differentiation of ESCs by upregulation of endogenous mechanical forces11. It is known that tumours are stiffened by extracellular matrix crosslinking11 and by actomyosin-driven collagen deposition12, suggesting that tumour cell differentiation might be regulated by the rigidity of substrates. Hence we hypothesize that if there were self-renewing tumorigenic cells among cancer cells, we might be able to use a mechanical strategy of soft substrates to select tumorigenic cells from a pool of cancer cells.

Soft 3D fibrin gels generate multicellular tumour spheroids

We prepared salmon fibrin gels (purified fibrinogen activated by thrombin) of different concentrations, which were mixed with dilute murine B16-F1 melanoma cells. Salmon fibrins are known for their nontoxicity and low immunogenicity and thus are excellent flexible scaffolds for transplanted cells in different animal species15,16. It has been reported that fibrin gels of 1, 4, 8 mg ml−1 correspond to 90, 420, 1,050 Pa in elastic stiffness17. We determined that fibrin gels of 90 Pa (1 mg ml−1) were the optimal gels for cancer cell proliferation and spheroid formation (Fig. 1a,b). About 1,250 B16-F1 cancer cells in 125 μl minimum essential medium (MEM), which had been trypsinized from conventional two-dimensional (2D) rigid dishes, were mixed with 125 μl fibrinogen solution (2 mg ml−1 fibrinogen, activated by 0.5 units of thrombin). The cells were trapped individually in the 3D fibrin gel and maintained in a MEM cell culture medium containing 10% fetal bovine serum (FBS). Inside the soft fibrin gel (90 Pa), about 100–150 spheroids colonies (~8–12% of all seeded cancer cells) formed and were maintained from day 2 up to day 6 (Fig. 1c); some cells at the bottom of the gel near the rigid dish exhibited spread morphology. In contrast, inside stiff gels (420 or 1,050 Pa), the spheroid colony number decreased dramatically to only a few as culture time increased, consistent with the data that the percentage of apoptotic colonies increased over time (Supplementary Fig. S1). Softer gels led to larger tumour spheroid size than stiffer gels (Fig. 1b,d). For each colony (that grew from a single cell) within fibrin gels of different stiffness, on day 5, there were more proliferating cells in the softest

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The stiffness of 3D fibrin gels with concentrations of 1 mg ml\(^{-1}\) apparent 90-Pa fibrin best promotes tumour growth. Mean ± s.e.m., n = 6 (for 90-Pa gels) or 3 (for 420- or 1,050-Pa gels) independent experiments. There are no significant differences between 90- and 1,050-Pa gels at day 1 (p > 0.2); there are significant differences at day 2 through day 6 (all p < 0.022). There are significant differences between 90- and 1,050-Pa gels at day 1 through day 6 (all p < 0.018). Between 420- and 1,050-Pa gels, there are differences only at day 1 (p = 0.037) and at day 5 (p = 0.00082). Colony size of the multicellular tumour spheroid as a function of culture time and fibrin stiffness. Apparently, 90-Pa fibrin best promotes tumour growth. Mean ± s.e.m., n = 6 (for 90-Pa gels) or 3 (for 420- or 1,050-Pa gels) independent experiments. The stiffness of 3D fibrin gels with concentrations of 1 mg ml\(^{-1}\), 4 mg ml\(^{-1}\) and 8 mg ml\(^{-1}\) is 90, 420, and 1,050 Pa, respectively. There are no significant differences at day 1 between 90- and 420-Pa gels (p = 0.37) and at day 5 (p = 0.27) or between 90- and 1,050-Pa gels (p = 0.33); from day 2 through day 6, there are significant differences between 90- and 420-Pa gels (p < 0.002 for all paired comparisons) and between 90- and 1,050-Pa gels (all p < 0.0012). The data in c and d are fitted by third-order polynomial functions (solid lines), the parameters of which are given in the Methods.
control B16-F1 cancer cells seem to spread and proliferate better on stiffer 2D polyacrylamide gels (Supplementary Fig. S10), consistent with published reports that stiffer substrates promote spreading and proliferation of differentiated cells and mesenchymal stem cells\(^{19}\), and contrary to what we observed inside the 3D fibrin gels, where the selected B16-F1 cells proliferated better in softer gels. Notably, the 2D soft substrates (of any matrix proteins) cannot be used for suspension growth-dependent cancer cells, making them non-ideal for selecting and growing soft-substrate conditioned cancer cells. Together, these findings suggest that 3D soft fibrin gels are unique in promoting multicellular tumour spheroid formation and growth.

### Tumorigenic ability of B16-F1 melanoma spheroids

We wondered whether the above B16-F1 melanoma spheroids formed in the 3D soft fibrin gel acquired more efficient tumorigenicity than those cultured on conventional 2D rigid dishes. For this purpose, single colonies were selected from 3D 90-Pa fibrin gels via pipetting, and each time 10 or 100 of such cells were subcutaneously inoculated to normal C57BL/6 mice. We found that 100, or even 10, of the above cells could form a subcutaneous melanoma with relatively high frequency (6/12 or 3/12, respectively). These cells grew very rapidly \(\textit{in vivo}\), with palpable tumours by 15 days for 100 injected cells and 21 days for 10 injected cells. Injecting the second-generation 3D soft fibrin gel cultured B16 cells into C57BL/6 mice also resulted in tumour formation at 10 or 100 cells per mouse. In contrast, subcutaneously injecting 100 of B16-F1 cells (dissociated directly from the conventional 2D rigid dishes) per mouse, formed no tumour (0/12); injecting 10 such cells per mouse could not form any tumour either (0/12) (Table 1). At least 10,000 B16 cells from rigid plastic dishes were required to form a tumour efficiently (Table 2); when we used limiting dilution assays\(^{\ast}\) to quantify the frequency of tumour formation for each condition—3D soft, 2D soft or plastic—we found that the highest frequency of tumour formation occurred in the 3D soft-fibrin gel condition (Table 2). A cardinal feature of malignant melanoma is its metastatic propensity to the lung. Using a well-characterized model of experimental lung metastasis by intravenously injecting cancer cells\(^{18}\), we delivered 100 or 10 of 3D-soft-gel cultured B16-F1 melanoma cells per mouse into C57BL/6 mice via the tail vein. The formation of metastatic tumours was examined on day 60. Tumour formation efficiency was relatively high: \(~40\%\) of mice formed tumours (5 out of 12 mice) for 100 cells, and \(~16\%\) of mice (2 out of 12 mice) formed tumours for 10 cells. In contrast, 10 or 100 control cells per mouse could not form any lung metastatic tumour (0/12) (Table 1). Most remarkably, B16-F1 tumour cells (from C57BL/6 mice) selected from the 3D soft fibrin gels were even capable of forming tumours in nonsyngenic BALB/c mice (Fig. 2a,b), although they grew slower than in SCID mice. To exclude the possibility that fibrin itself might play a role in the tumorigenicity of the cancer cells, control B16-F1 cells were mixed with the soft 90-Pa fibrin gel and treated the same way as the above B16-F1 cells cultured in the 90-Pa 3D fibrin gel. One hundred cells were then subcutaneously or intravenously injected into C57BL/6 mice with different cell numbers. CD133\(^{\ast}\)/CD44\(^{\ast}\): the B16-F1 cells were cultured for 5 days on rigid plastic and labelled with FITC-conjugated anti-CD133 and PE-conjugated anti-CD44 antibodies, and then sorted by FACS. The tumour formations were counted as a function of injected cell number.

### Uptregulation of stem cell markers in B16-F1 spheroid cells

The above \(\textit{in vivo}\) tumour formation data suggest that cells within the spheroids formed in the soft 3D fibrin gel may share some features of a stem cell. To further test this idea, B16-F1 melanoma cells were trapped in the 90-Pa fibrin gel and cultured for five days. The formed spheroids were picked out and the cells were used for ribonucleic acid (RNA) isolation. A panel of stem cell markers—Oct3/4, Nanog, CD133, nestin, Bmi-1 and c-kit—were determined by reverse transcription polymerase chain reaction (RT-PCR). The expression of Oct3/4 or Nanog was not detected in either 3D fibrin-gel- or 2D-rigid-dish-cultured B16-F1 cells (Fig. 3a), but CD133, nestin, Bmi-1 and c-kit were up-regulated, when compared with the controls (Fig. 3a). In line with the RT-PCR result, upregulation of nestin, Bmi-1 and c-kit was further confirmed with real-time RT-PCR, although the increase in CD133 was not significant (Fig. 3c). Telomerase enzyme activity is known to be expressed in ESCs and stem-cell-like cancer cells\(^{19}\). When we analysed the expression of murine telomerase reverse transcriptase subunit (mTERT), the catalytic component of telomerase, we found that mTERT was up-regulated in the cells from the soft 3D fibrin gel

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**Table 1** | Tumorigenicity of 3D-fibrin-gel-cultured B16-F1 cells in C57BL/6 mice.

| Tumour formation of B16-F1 cells in C57BL/6 mice | Mouse model | 3D soft fibrin | 2D soft fibrin | Plastic |
|-----------------------------------------------|-------------|---------------|---------------|--------|
| B16-F1 (100 cells)                            | 3D          | 6/12          | 5/12          | s.c.*  |
| Control                                       | 0/12        | 0/12          | 0/12          | i.v.*  |
| B16-F1 (10 cells)                             | 3D          | 3/12          | 2/12          | s.c.*  |
| Control                                       | 0/12        | 0/12          | 0/12          | i.v.*  |

*s.c.: subcutaneous injection; i.v.: intravenous injection.

**Table 2** | 3D soft fibrin gels promote more efficient tumorigenicity.

| Tumour formation of B16-F1 cells in C57BL/6 mice | Cell number | 2D soft fibrin | 3D soft fibrin | Plastic | CD133\(^{\ast}\) | CD44\(^{\ast}\) |
|-------------------------------------------------|-------------|---------------|---------------|--------|----------------|----------------|
| B16-F1 cells were cultured on 2D soft fibrin gels (2D soft fibrin) (90 Pa), in 3D soft fibrin gels (3D soft fibrin) (90 Pa) or on rigid plastic (Plastic), all for 5 days. They were then subcutaneously injected into C57BL/6 mice with different cell numbers. CD133\(^{\ast}\)/CD44\(^{\ast}\): the B16-F1 cells were cultured for 5 days on rigid plastic and labelled with FITC-conjugated anti-CD133 and PE-conjugated anti-CD44 antibodies, and then sorted by FACS. The tumour formations were counted as a function of injected cell number.

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\(^{\ast}\)s.c.: subcutaneous injection; i.v.: intravenous injection.
Figure 2 | Tumour metastasis of 3D-cultured B16-F1 cells in lung tissue of BALB/c mice. a. Ten cells from B16-F1 tumour spheroids formed in soft 3D fibrin gels after 5-day culture were injected into the tail veins of BALB/c mice. Mice were euthanized after two months of injection and the lung tissue image was recorded. B16-F1 cells cultured in 2D rigid dishes were used as a control. In the experimental group, three out of four mice formed lung tumours (top panel), whereas in the control group, no tumours were formed (bottom panel). The results shown are representative from three independent experiments. b. Lung tissues from mice in the above 3D group exhibit different patterns from those in the control group. Left and right images are lung tissues (stained with H&E) from two experimental mice (top; numerous tumour cells) and from two control mice (bottom; normal alveoli). All images were taken at ×10 magnification.

As well as Oct3/4 and Nanog, we examined expression of three other self-renewal markers: c-myc, Rex-1 and Sox2 in B16-F1 cells. Rex-1 was not detected and c-myc was equally expressed in the cells from the 3D soft fibrin gels and from the rigid plastic. Interestingly, Sox2 was only expressed by the cells from 3D soft fibrin gels (Supplementary Fig. S12), suggesting that this unique microenvironment might be promoting self-renewal of these tumorigenic cells via Sox2. Moreover, silencing Sox2, c-kit, Nestin, or Bmi-1 in cells on 2D soft fibrin gels (90 Pa) via small interfering RNA (siRNA) transfection promoted spreading of the round colony (Supplementary Fig. S13). As published reports have shown that colony spreading is necessary for the inhibition of self-renewal of ESCs and for the onset of differentiation of ESCs (refs 10,11), the results suggest that these self-renewal markers, especially Sox2, are required for the phenotypes of the cells in soft fibrin gels.

It is known that ‘cancer stem cells’ are more resistant to chemotherapeutic drug-induced apoptosis. To determine if these 3D-fibrin-gel-selected cells are more drug-resistant, different concentrations of doxorubicin or cisplatin were added during the last 18 h of the 5-day culture in the 90-Pa 3D fibrin gels. In line with the expression of stem-cell-associated markers, B16-F1 cells from 3D fibrin gels were more resistant to apoptosis, compared with those from the 2D rigid dish (Fig. 3e and Supplementary Fig. S14).

To further test the possibility of the self-renewing capacity of these tumour-repopulating cells, we conducted serial transplantation in mice. 100,000 B16-F1 melanoma cells, isolated from the primary tumour that was formed by injecting 100 B16 cells from 3D soft fibrin gels, also generated tumours in C57BL/6 mice. Such serial transplantation could be successively carried out for at least three generations. Together, these data suggest that the
cells from spheroids formed in the 3D soft fibrin gel acquire self-renewing capacities.

**Unique biophysical properties of tumorigenic cells**

The importance of substrate rigidity in stem cell differentiation and self-renewal is becoming increasingly evident. To determine the biophysical mechanisms of B16-F1 cells possessing stem-cell-like features and tumorigenicity after being cultured within the soft 3D fibrin gel, we re-plated these 3D-fibrin gel cultured tumorigenic cells (from spheroids formed in 3D soft fibrin gels) on a 2D flexible substrate and quantified their mechanical stiffness and tractions. Their intrinsic cell stiffness (defined as stiffness on rigid surface) was ~0.05 kPa on the rigid glass, about 25% of the stiffness of the control cancer cell (~0.2 kPa; Fig. 4a). Notably, these tumorigenic cells did not stiffen much with substrate stiffness (Fig. 4a), but their tractions increased dramatically (by a factor of ~3) when substrate stiffness increased from 0.6 to 8 kPa, similar to a recent finding in mouse ESCs (ref. 22). In contrast, both the stiffness and tractions of control cancer cells increased proportionally with substrate stiffness (Fig. 4c). Together with the data of low cell stiffness, round morphology on 2D rigid substrates, and the formation of spheroid colonies within soft 3D gels, this suggests that these tumorigenic cells possess some key phenotypic and biophysical features of ESCs, without expressing the ESC markers Oct3/4 and Nanog (Fig. 3a).

To further explore the cellular and molecular mechanisms of soft-fibrin-gel-selected cells, we measured the expression of \( \alpha, \beta_1 \) integrin, which binds to fibrin, and \( \alpha, \beta_3 \) integrin expression between fibrin-gel-cultured cells and control cells (Supplementary Fig. S16). However, B16-F1 cells depended on \( \alpha, \beta_3 \) integrin to attach and grow in the 3D soft fibrin gels, as a blocking antibody to \( \alpha, \beta_3 \), but not a blocking antibody to \( \beta_1 \) (\( \alpha_1/\alpha_2/\beta_1 \) engaging collagen-I; ref. 24), completely blocked spheroid colony formation and growth in a dose-dependent manner (Supplementary Fig. S17). This result suggests that, although these cells may synthesize and secrete other matrix proteins during the 5-day culturing period, they primarily depend on fibrin-\( \alpha, \beta_3 \) integrin interactions for survival and growth. Furthermore, inhibition of Rho-associated protein kinase (ROCK) with Y-27632 decreased spheroid colony number and colony growth for both 3D and 2D soft fibrin gels in a dose-dependent manner (Supplementary Fig. S18), suggesting that RhoA-dependent cell contractility might

### Figure 3 | Upregulation of stem-cell-associated genes in B16-F1 spheroid cells cultured in 3D fibrin gels.

- **a.** Stem-cell-marker expression in B16-F1 spheroid cells. Total mRNA of B16-F1 spheroid cells at day 5 was extracted and used for the detection of Nanog, Oct3/4, CD133, nestin, Bmi-1 and c-kit mRNA expression by RT-PCR. B16-F1 cells cultured in 2D rigid dishes were used as a control. Three independent experiments showed similar results.
- **b.** mTERT expression of B16-F1 spheroid cells. mTERT mRNA expression was measured by RT-PCR; representative images of three independent experiments.
- **c.** The expression of stem cell markers and mTERT in B16-F1 cells was quantified by real-time PCR. The same mRNA sample of B16-F1 tumour spheroid cells as above was used. B16-F1 cells cultured in 2D rigid dishes were used as control. Mean ± s.e.m.; *p < 0.05, compared with control cells.
- **d.** The expression of stem cell markers and mTERT in B16-F1 cells was quantified by real-time PCR. The same mRNA sample of B16-F1 tumour spheroid cells as above was used. B16-F1 cells cultured in 2D rigid dishes were used as control. Mean ± s.e.m.; n = 3 independent experiments; *p < 0.05, compared with control cells.
- **e.** Apoptotic analysis of doxorubicin-treated 3D B16-F1 cells. Different concentrations of doxorubicin were added to the B16-F1 cell culture medium during the last 18 h of the 5-day culture in the 90-Pa 3D fibrin gels or conventional 2D rigid dishes. Cells were collected and stained with FITC-conjugated Annexin-V for apoptotic detection by flow cytometry. Mean ± s.e.m., Apoptotic rate (%).
be associated with the selection and growth of these tumorigenic cells in the 3D soft fibrin gels.

The establishment of robust and reliable methods to identify and isolate tumour-repopulating cells remains a major challenge for cancer research. Although a number of cell surface markers have been proved useful for the isolation of subsets enriched for ‘stem-cell-like cancer cells’, the use of those surface markers is controversial and their relevance to tumour-repopulating cancer cells is not clear. In the present study, we introduced an alternative mechanical method to select and grow tumour-repopulating cells from a pool of cancer cells, without using surface-marker labelling or other intrusive methods that might alter cell functions or phenotypes. By culturing cancer cells within very soft 3D fibrin gels, which are as soft as the intrinsic stiffness of those cancer cells, we have demonstrated that the selected cells in the form of spheroids exhibit highly efficient tumorigenicity and are able to survive and form tumours in syngeneic or SCID mice, either injected subcutaneously or intravenously. Most strikingly, only as few as ten such cells are needed to form tumours in the lungs of normal wild-type non-syngeneic mice, suggesting that these tumorigenic cells from multicellular tumour spheroids are able to survive the harsh environment in the lung and grow multiple large tumour colonies in vivo. The soft-fibrin-gel-based method described in this study possesses some advantages over conventional stem cell surface marker-based methods, as the sorted CD133<sup>+</sup>CD44<sup>+</sup> B16 cells could not form a tumour with 100 cells; even when 1,000 cells were transplanted only one third of the mice developed tumours (Table 2). As well as selection and enrichment, our data suggest the possibility of ‘priming’. If we assume that ~10% of the cancer cells in the general population are tumour-repopulating cells (based on our in vitro data in colony formation in Fig. 1), then one would predict that 100 cells from the plastic should be sufficient to form tumours, because 10 cells from the 3D soft fibrin gels are sufficient to generate tumours. However, we find that 1,000 cells from the rigid plastic cannot form any tumour at all (0 out of 6 mice) and that it takes 10,000 cells to be 50% efficient (3 out of 6 mice) in generating tumours, suggesting that there is some ‘priming’ by the 3D soft fibrin gels. Consistent with this interpretation, B16-F1 cells from the 3D soft fibrin gels, but not from the rigid plastic, express Sox2, which might be essential for self-renewal and growth of these tumour-repopulating cells in vivo, because knocking down Sox2 leads to differentiation of these soft fibrin cultured, self-renewing B16-F1 cells. Our findings that the B16-F1 melanoma cells from 3D soft fibrin gels can form efficient tumours in wild-type nonsyngeneic mice might also be partially explained by a finding that in vitro melanoma spheroid cells do elicit a lower allogeneic response from immune cells<sup>25</sup>. In contrast, as many as >20,000 cells are needed to generate tumours in immunodeficient IL-2<sup>−/−</sup> mice from the tumour-initiating cells<sup>9</sup>. Although in vitro transformation of human fibroblasts can generate a multipotent cell type with stem-cell-like cancer cell properties, it still takes at least 100 cells to generate tumours in NOD-SCID IL-2<sup>−/−</sup> mice<sup>66</sup>

We further investigated the mechanism of how these tumorigenic cells manage to maintain long-term survival and growth in vivo. An in vitro trans-well study reveals no difference in transmigration through 8-µm membrane pores between 3D-fibrin-gel-cultured cells and control cells (Supplementary Fig. S19), suggesting that the high colonization efficiency and long-term survival of these soft tumorigenic cells cannot be recapitulated by the models of the transwell assays, consistent with the in vivo data that both control cells and 3D-soft-fibrin-gel-cultured cells gain access to the lung from the blood within a few hours post-injection (Supplementary Fig. S11). However, the softness (or the high cell deformability, which is the inverse of cell stiffness) of these tumorigenic cells facilitates them (less energy needed) to change cell shape and thus to penetrate the endothelial monolayer in the lung and move through the dense extracellular matrices of the colonized tissues. This interpretation is supported by our finding that these tumorigenic cells do not stiffen in response to a much stiffer substrate (Fig. 4a; as they might encounter in vivo), similar to the behaviour found in mouse ESCs (ref. 22). Importantly, these cells do dramatically elevate their tractional forces with substrate stiffness (Fig. 4b), which is necessary for the tumorigenic cells to invade or to move through the dense extracellular matrices. Although cells from both the 3D soft fibrin gels and control cancer cells can reach the lung tissue, whether there are differences in the exact location of these cells in the lung microenvironment and in the local mechanical properties of the tissue is not clear at this time. It also remains to be determined what is the relationship between the softness of these soft-fibrin-cultured cells and self-renewing gene expression and long-term growth in vivo. Furthermore, the anti-apoptotic feature of these 3D-fibrin-gel-selected cells (Fig. 3e) may also contribute to the long-term survival of these tumour-repopulating cells in the lungs of mice. Recently, it has been shown that Janus kinase 1 (JAK1) and ROCK mediate high contractility of the round amoeboid-like cancer cells, which plays a critical role at the leading edge of the collective migration of invading cancer cells<sup>27</sup>. Thus it will be interesting to determine the signalling pathways during migration and invasion of these soft but high force generating
tumorigenic cells. Such high tumorigenicity efficiency cannot be ascribed to the specific effect of the fibrin gel in shielding the cancer cells from the animal host immune responses, because a mixture of fibrin gel with control cancer cells did not elevate the efficiency of tumorigenicity. Our findings show that soft 3D fibrin gels are much better than soft 3D collagen-1 gels in selecting tumorigenic cells and promoting tumorigenic cell proliferation. This suggests that, although the stiffness of 3D substrates is a critical factor in determining whether a tumour spheroid colony forms or not, other factors such as integrin subsets, polymer size and orientation are further important factors in regulating tumorigenic cell proliferation rates, invasion and metastasizing potential.48,49 Also, it is known that salmon fibrin, besides having a unique nonlinear elasticity behaviour,5 poses some unique advantages: salmon fibrin has been successfully used in several animal models of neuronal wound healing, and are probably low in immunogenicity and low in virus infection propensity, suggesting that salmon fibrin gels might be used in vivo physiologic conditions of different animal species to study cancer progression and metastasis.

Numerous reports show that fibrin is present in connective tissue stroma in human malignant tumours, where it fibrinogen increases the survival and metastatic potential of circulating tumour cells,50,51 that fibrin–fibronection complexes promote lung metastasis,52 and that fibrinogen depletion down-regulates pulmonary tumour formation in wild-type mice.53 In our study, fibrin was confirmed to be expressed in the stroma of B16-F1 melanoma (Supplementary Fig. S20). Furthermore, we found that the activity of AKT1/2 kinase (AKT is also known as protein kinase B), an important signalling molecule involved in melanoma cell proliferation and migration, was elevated in B16-F1 cells from 3D soft fibrin gels, compared to that from the rigid plastic or to that from the 3D soft collagen gels (Supplementary Fig. S21). Unlike the cells from the rigid plastic or from the 3D collagen gels, the cells from the 3D soft fibrin gels do not change their AKT activity after treatment of epidermal growth factor (EGF). Such AKT1/2 pathways seem to be critical for B16 spheroid development in 3D soft fibrin gels, as a selective AKT1/2 inhibitor suppressed the spheroid growth (Supplementary Fig. S22). Therefore, the above-mentioned reports, together with our current study, suggest that fibrin may play a significant role in cancer that has been previously under-appreciated.

It is of considerable interest that the optimal stiffness of 3D fibrin gel for tumorigenic cell proliferation and spheroid formation is ~90 Pa (~30 Pa in shear modulus), which is less than 1/6 of the control B16-F1 cancer cell stiffness, and ~1/2 of the stiffness of tumorigenic cells cultured in the 3D fibrin gels (Fig. 4). These findings suggest that these tumorigenic cells may prefer for their expansion a soft microenvironment with characteristics that resemble those of the soft substrates that mouse ESCs promote for their self-renewal and pluripotency.6,10,11 Our results are consistent with a recent report that soft 3D collagen gel substrates are more conducive than stiff ones in facilitating tumour spheroid formation,55 supporting the postulate of a physical microenvironment barrier (for example, extracellular matrix) in the inhibition of cancer progression.6,16 The discrepancies between our findings in these tumorigenic cells and the report that the stiffening of the extracellular matrix facilitates mammary epithelial cell transformation and tumour progression12 might lie in the different substrate-rigidity-dependent growth behaviours between the tumour-repopulating soft tumorigenic cells and the stiffer differentiated cancer cells.

Our findings that ~10% of mouse B16-F1 melanoma cells have extremely high tumorigenic ability are consistent with a previous report6 on ~25% of human melanoma cells with tumorigenic ability. However, the high tumorigenicity efficiency of those single cells is only achieved in IL-2γ−/− NOD/SCID mice. In contrast, our present study demonstrates that healthy syngeneic mice, and even non-syngeneic mice, develop tumours after implantation of a few cells selected from 3D soft fibrin gels. Our present work suggests that it is possible to decrease the stochastic nature of state transitions between stem-like and non-stem-like cancer cells when the mechanical properties of their microenvironment are better defined and controlled. The findings that tumorigenic cells are soft and generate low tractions are in accord with recent reports on the role of mechanical forces (exogenous or endogenous) in stem cell fate and in tumour progression12,13,14. A recent report shows that 2D soft substrates do not select a rigidity-independent subgroup from rigidity-dependent cells.57 Our 3D soft substrates, on the other hand, do select a tumorigenic subgroup from a pool of cancer cells. Recently it was shown that a transcriptional coactivator with PDZ-binding motif (TAZ) is necessary for self-renewal and tumour initiation in breast cancer cells,58 and that the Yes-associated protein (YAP)/TAZ seems to be important in the matrix-rigidity-dependent differentiation of mesenchymal stem cells.57 It will be interesting to see if TAZ plays a role in the tumorigenicity of the tumour-repopulating cells from the 3D soft fibrin gels. It still remains to be elucidated how 3D-soft-fibrin-selected tumour-repopulating cells can survive and generate efficient tumour colonization6 locally and at distant organs.

In summary, these data show that a 3D fibrin gel, by virtue of its softness relative to the cancer cell stiffness, can control differentiation behaviours and proliferation rates of tumour cell subsets. This mechanical method is useful in culturing, selecting, and growing tumorigenic cells from cancer cell lines that are capable of forming efficient tumours in syngeneic, SCID, or normal wild-type non-syngeneic mice with as few as 10 cells, independent of stem cell markers. Thus, the present approach not only provides a useful platform for understanding the underlying mechanisms of tumorigenicity and metastasis14,15, but also opens a new avenue in the study of tumour-repopulating cancer cells.

Methods

Ethics statement. The animal studies were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH). The protocol was approved by the Animal Care and Use Committee of Tongji Medical College. All efforts were made to minimize suffering.

The clinical tumour specimens were acquired from patients with breast cancer or with T-cell acute lymphoblastic leukaemia, as approved by the Ethical Committee of the Medical Faculty of Tongji Medical College. Informed consent was obtained, in accordance with the Declaration of Helsinki, from all subjects. The specimens were de-identified before being given to the experimentalists.

Animals and cell lines. Six-week-old C57BL/6 mice, SCID mice, nude mice, and BALB/c mice, all female, were purchased from Wuhan University Center for Animal Experiment. All animals received humane care in compliance with the ‘Principles of Laboratory Animal Care’ formulated by the National Society of Medical Research and the guide for the US NIH. The protocol was approved by the Animal Care and Use Committee of Wuhan University. Human ovarian cancer cell line A2780, human liver carcinoma cell line HepG2, murine lymphoma cell line EL4, and murine hepatocarcinoma cell line H22, murine lymphoblast-like mastocytoma cell line P815 were purchased from China Center for Type Culture Collection; murine melanoma cell line B16-F1 were purchased from either China Center for Type Culture Collection or ATCC.

Conventional 2D-rigid-dish and 3D-fibrin-gel cell culture of tumour cells. For the conventional 2D cell culture, B16-F1 cells were maintained in a rigid dish with MEM cell culture medium supplemented with 10% FBS (Invitrogen) at 37 °C with 5% CO2. For the 3D-fibrin-gel cell culture, salmon fibrinogen and thrombin were purchased from Searum Holdings. Detailed methods are described in the Supplementary Information.

Reverse transcription-PCR and real-time PCR analysis. Total RNA of B16 cell spheroids from 3D-fibrin-gel culture were extracted using Trizol reagent according to the supplier’s instruction (Invitrogen). The relative quantity of mRNA was determined by reverse transcription-PCR (RT-PCR; 30 cycles, One-step RT-PCR kit, purchased from Qiagen). Oct3/4, Nanog, CD133, nestin, Bmi-1, c-kit, TERT, c-myc, Rex-1, Sox-2, integrins αv, β3, α6, and β1, mRNA expressions were examined. The mRNA of glyceraldehyde-3-phosphate dehydrogenase was used as the internal control. The primer sequences are shown in Table S2.
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