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INNOVATION

Mapping normal and cancer cell signalling networks: towards single-cell proteomics

Jonathan M. Irish, Nikesh Kotecha and Garry P. Nolan

Abstract | Oncogenesis and tumour progression are supported by alterations in cell signalling. Using flow cytometry, it is now possible to track and analyse signalling events in individual cancer cells. Data from this type of analysis can be used to create a network map of signalling in each cell and to link specific signalling profiles with clinical outcomes. This form of 'single-cell proteomics' can identify pathways that are activated in therapy-resistant cells and can provide biomarkers for cancer diagnosis and for determining patient prognosis.

Alterations in signalling result in increased survival and proliferation of cancer cells, as well as increasing immune evasion by cancer cells. Although a wide variety of genetic and epigenetic events contribute to these alterations, it has been challenging to gain an overall picture of the common effects that these changes have on the entire signalling network. A 'network-level' view of signalling in normal and cancer cells is therefore needed to identify shared features of malignant cells. Flow cytometry, which simultaneously quantifies multiple properties of individual cells, is well suited to this task because a 'map' of the signalling network can be derived for each cell in a mixed population and compared with other cellular features. This 'single-cell resolution' and the multi-parameter nature of the data can be used to distinguish signalling maps of cancer cells from non-tumour cells present in patient samples. It is also possible to identify cancer cell subsets based on their signalling maps. By relating changes in cancer signalling networks to patient outcome, cancer cell signalling can be used to

Table 1 | **Determining phenotypes of individual cancer cells**

Cell property*	Example flow-cytometry method	References
Differentiation and lineage determination	Antibodies against KIT, CD34 (stem cells), CD38 or CD20, and other CD antigens	29–32
DNA content (aneuploidy, DNA fragmentation)	Propidium iodide, ethidium monoazide or 7-actinomycin D staining of DNA	30,33
RNA content (quiescence)	Pyronin Y staining of RNA	30
Cell-cycle stage	Antibodies against cyclin D, cyclin A, cyclin B1 or cyclin E; phosphorylated form of histone H3 (M phase)	30,34,35
Proliferation	Bromodeoxyuridine staining of DNA replication; antibodies against proliferating cell nuclear antigen; antibodies against Ki67; carboxyfluorescein diacetate succinimidyl ester dye	30,31,36,37
Oncogene expression	Antibodies against BCL2, MYC or Ras	31,38–40
Mutations	Antibodies against mutant p53 or $HRAS^{V12}$	41,42
Tumour-suppressor activity	Antibodies against p53 or p21 (also known as WAF1) promoter activity based on expression of green fluorescent protein (p53R–GFP system)‡; antibodies against the phosphorylated form of p53‡	23,41
Apoptosis	Antibodies against caspase 3 cleavage products	44
Cell-membrane changes	AnnexinV staining for extracellular phosphatidylserine exposure, which occurs on apoptotic cells	44
Redox state	Dichlorofluorescein diacetate staining, which is a measure of oxidation; monobromobimane staining, which is a measure of glutathione; lipophilic fluorochrome dihexaoxacarbocyanine iodide staining, which is a measure of mitochondrial membrane potential	44–46
Tumour antigens	Antibodies against B- or T-cell receptor idiotype; tetramers against tumour antigen-specific T cells (for example, against tyrosinase)	5,47,48
Signalling activity	Antibodies against phosphorylated signal transducer and activator of transcription 5, extracellular-regulated kinases 1 and 2, and many others; indo-1 staining for Ca^{2+} flux; antibodies against interleukin 12, interferon-γ or other cytokines	4,48–50

*Up to 17 such properties can be simultaneously measured in every cell using flow cytometry[3]. ‡Applied to cancer cell flow cytometry by J.M.I. (unpublished observations). CD, cluster of differentiation.

determine if a tumour is aggressive or will respond to certain therapies. How can flow cytometry be used to characterize altered signalling network mechanisms in primary cancer cells directly? What can be learned about pathogenesis from these signalling profiles, and how can this information be applied to improve clinical outcomes?

Cancer cell cytometry

Flow cytometry has been widely used by immunologists and cancer biologists for many years to distinguish different cell types in mixed populations, based on the expression of cellular markers. Commonly, cells that express a protein of interest are detected using a dye conjugated to an antibody that specifically binds that protein and increases cell fluorescence. In addition to tracking populations by protein expression, flow cytometry can quantify many other cancer cell properties (TABLE 1), such as phosphorylation levels, which can be used to determine signalling activity[1,2]. For analysis of cell biology, cells are usually fixed and permeabilized, allowing molecules from cellular compartments such as the cytoplasm or nucleus to be detected. Cells are streamed in a single file past a detector at a rate of many tens of thousands of cells per second, and multiple properties of interest are measured for each cell (FIG. 1). Because cells must be dissociated for analysis, flow cytometry has primarily been applied to study haematological cancers. The current state of the art supports detection of 17 properties simultaneously in individual cells in a high-throughput manner[3].

This 'multi-parameter' aspect of flow cytometry — the ability to measure multiple biomarkers per cell — makes it an especially useful tool for understanding the biology of heterogeneous populations of cells, such as those found in tumour samples from patients. These samples generally include a mixture of host cancer cells and host non-cancer cells. In addition, cancer cells are genetically unstable, and multiple subpopulations of cancer cells with differences in signalling activity can arise[4]. Flow cytometry capitalizes on the molecular features of these subsets and uses them as biomarkers to identify different tumour types or properties (TABLE 1). For example, features of B-lymphoma, such as lineage and B-cell-receptor idiotype[5], can be used to distinguish populations of tumour B cells from infiltrating non-tumour B and T cells within an individual tumour biopsy specimen. In comparing signalling profiles of cell subsets within a single tumour sample, greater than normal extracellular-regulated kinase 1 (ERK1)/ERK2 signalling responses are restricted to the tumour B cells, whereas normal or suppressed ERK1/ERK2 signalling takes place in the infiltrating non-tumour cells of B or T lineages (J.M.I., G.P.N. and R. Levy, unpublished observations). Such biological and mechanistic observations can be correlated with cellular phenotype or patient outcomes, creating powerful tools for studying cancer and for identifying therapeutic strategies.

Mapping cell-signalling networks

As the technology to measure signalling has developed, so has a common language to describe cell signalling networks (BOX 1). The terms that we use to describe cancer signalling networks are adapted from graph theory[6] and are used in computational modelling of biological networks[7,8]. A measured event that corresponds to a change of state in a signalling molecule is called a 'signalling node'. Biochemical events, such as phosphorylation, are understood in advance to be mechanisms of activating or inactivating signalling nodes. One practical reason for this general term 'node' is that signals in cells are conveyed by many biochemical events other than phosphorylation, including acetylation, ubiquitylation, proteolytic cleavage, and changes in localization, conformation and abundance. The structure of connections between signalling nodes is commonly referred to as a 'map' of the signalling network. Because the relationships among nodes are thought to determine cell behaviour, mapping the altered connections among nodes in a signalling network could

indicate mechanisms that support the continued survival and proliferation of cancer cells.

The measured state (activity or inactivity) of a signalling node under a specific set of conditions is called the 'node state' (BOX 1). Clinically relevant signalling-node states might differ among tumour types, and factors that affect the choice of nodes and states in cancer can be examined in detail. In a signalling analysis, the unstimulated or minimally perturbed state measured is referred to as the 'basal state'. For most signalling nodes in resting normal cells, the basal state is usually 'inactive'. However, in resting cancer cells, the basal state of a node might be 'active' because of constitutive activation of signalling pathways.

A significant amount of information about the signalling network can be gained by tracking signalling activity as it occurs under various conditions. This is achieved experimentally by exposing live cells to different external cues (inhibitors, stimulations, and combinations of both), quantifying the state of each signalling node, and then comparing the signalling activity to the basal state (FIG. 1). The primary advantage of this approach is the ability to measure signalling network properties that cannot be detected in resting cells. Some important signalling network properties that are not seen in resting cells include failure of the signalling network to become activated following stimulation, hypersensitivity of the signalling network to stimulation, and differences in which signalling nodes are activated following a particular upstream event (sometimes called 'rewiring' or 'crosstalk'). A key feature of this technique is that individual nodal elements are linked to each other in a network map by multiple stimulations, providing a more dynamic understanding of how information is processed by the system.

The high-throughput nature of flow cytometry allows for the measurement of several signalling nodes and several states for each sample. In mapping signalling profiles of acute myeloid leukaemia (AML) cells[4], 36 node states were measured by combining 6 stimulation conditions with 6 detectable signalling nodes. These node states were measured in at least 50,000 individual cells from 30 different samples taken from patients with AML, resulting in millions of cell-signalling maps. Measuring tens to hundreds of thousands of cells in each sample provides statistical confidence in small populations (for example, a subset that comprises only 1% of cells in a sample), and is a relatively small number of cells to require for an assay. A sample of 5 million cells from a patient is sufficient to map 100 signalling node states (10 nodes x 10 states) while simultaneously determining lineage and oncogene-expression patterns of each cell. With existing 96-well format technology, this analysis can be performed from start to finish on tens of patient samples in a matter of hours.

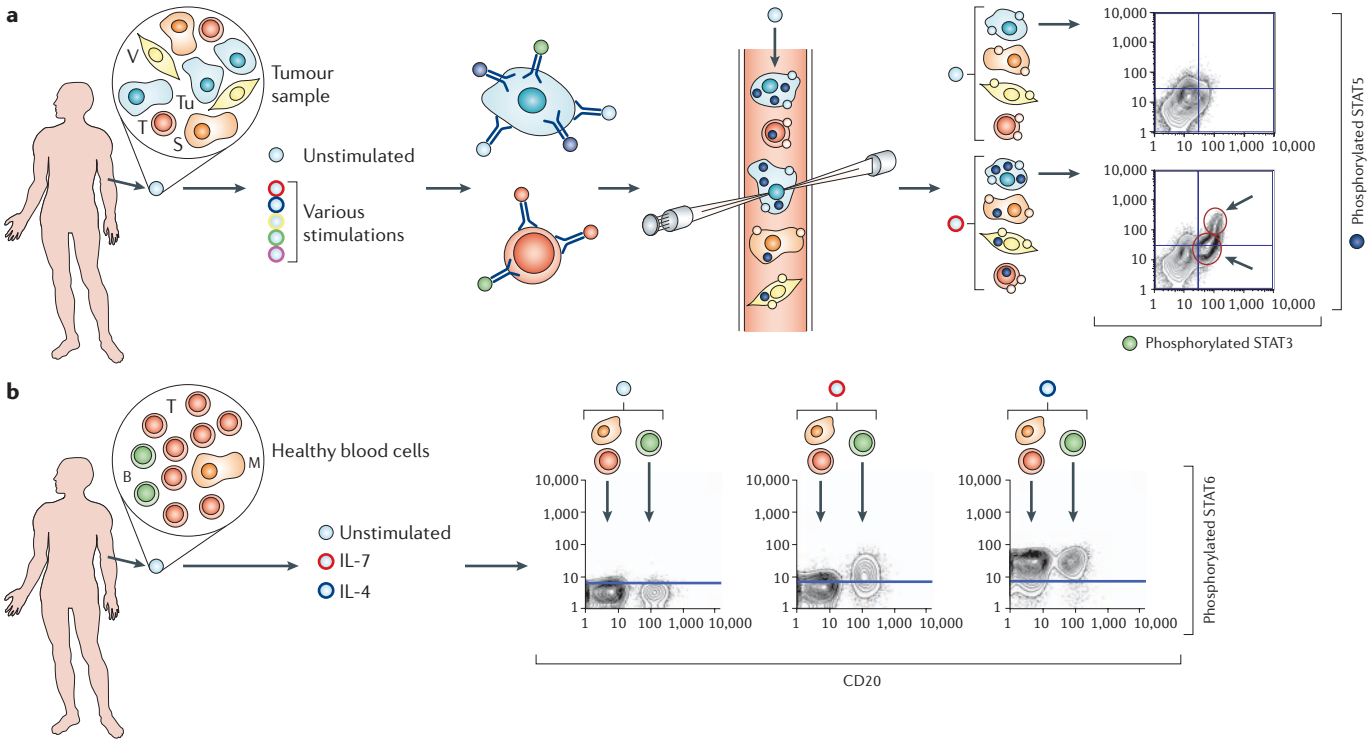

Figure 1 | **Individual-cell analysis of signalling. a** | Tumour cells that have been isolated from a patient are treated with different environmental cues or therapeutic agents as a way to identify which signalling networks are active. It is possible to study cancer cells from the tumour (Tu), stromal cells (S), cells of the vasculature (V), or immune cells such as T cells (T). Using flow cytometry, each cell in the sample can be tracked and the per-cell phosphorylation levels of multiple proteins (for example, signal transducer and activator of transcription (STAT) 3 and STAT5) can be determined using fluorophore-conjugated antibodies in cells treated with different stimuli (red, blue, yellow, green and pink circles). The cells are analysed individually by the detector. Phosphoprotein levels of each cell are compared using histograms in which the axes indicate the levels of STAT3 phosphorylation in cells (*x* axis), and STAT5 (*y* axis). The arrows point to a population of stimulated cells with high levels of phosphorylation of both STAT3 and STAT5. **b** | The same technique can be used to study signalling in subsets of normal primary cells, such as T cells (T), B cells (B) or monocytes (M) after treatment with various stimuli, such as interleukin (IL)-7 (red circles) or IL-4 (blue circles). Phosphorylation levels of STAT6 (*y* axis) were compared in human blood cell subsets, which were identified based on expression of the B-cell lineage marker CD20 (*x* axis). B cells treated with IL-7 did not phosphorylate STAT6 to the extent of B cells treated with IL-4 (far right histogram). A better understanding of normal signalling in primary cells is crucial to understanding altered signalling in cancer cells.

Box 1 | **Terminology used in studying signalling networks**

Signalling node
A step in a signalling pathway that corresponds to a biochemical event. Signalling inputs (for example, ligand binding) and outputs (for example, gene expression) are also considered nodes.
For example: interferon-γ (IFNγ) activates a signalling node that involves phosphorylation of signal transducer and activator of transcription (STAT) 1 at Y701 (pSTAT1-Y701). This normally results in STAT1 dimerization, nuclear translocation and transcriptional activity (FIG. 3a).

Node state
A property of a node (activity or inactivity) under a certain set of conditions that are specific to details of the experimental system (for example, cell type or stimulation time).
For example: in studying STAT1, one node state is whether Y701 is phosphorylated or not 15 minutes after IFNγ-stimulation of cells.

Signalling profile
A collection of signalling features that either defines a group of cells or is specific to a group of cells. This profile can then be used to characterize specific patient samples or populations of cells.
For example: the signalling profile of cells from patients with acute myeloid leukaemia that are resistant to therapy included a failure of STAT1 to become activated in response to IFNγ stimulation, and potentiated STAT5, STAT3 and extracellular-regulated kinase 1 (ERK1)/ERK2 signalling responses.

Cell signalling in cancer

Although the technology and language associated with the study of signalling networks is relatively new, the importance of cell signalling in cancer is well established. Alterations to genes that encode signalling molecules and their regulators are commonly observed in many types of cancer and are known to support cancer cells by providing hallmark characteristics[9,10], such as evasion of cell death and self-sufficiency in proliferative potential (TABLE 2). So, it is not surprising that patient outcome can sometimes be associated with a specific mutation in a signalling factor (BOX 2). A central hypothesis of the signalling profile approach is that patients whose cancer cells display common signalling mechanisms will have similar clinical outcomes (FIG. 2).

One starting point in the study of cancer signalling networks might be to characterize how a clinically relevant mutation in a signalling protein (BOX 2) impacts on a signalling profile of a cancer cell. This can be done by comparing samples of cells that express mutant and wild-type versions of a gene. Once the signalling network alterations that are associated with a specific mutation are identified, the analysis can be extended to look for the same profile in samples with no known mutations. This approach could identify new mutations that result in a similar cell phenotype. In primary cancer cells, different genetic defects might result in the activation of the same signalling node, resulting in similar profiles. Identification of such focal signalling nodes is an important aspect of anticancer drug development, as these signalling nodes are good therapeutic targets for patients whose tumours arise from different genetic alterations. The effectiveness of the tyrosine-kinase inhibitor imatinib (Glivec), which is successfully used to treat patients with chronic myeloid leukaemia (CML)[11], supports the idea that inhibitors of certain signalling nodes that are active in different cancers make effective therapeutics[12].

Some signalling molecules, such as AKT and ERK1/ERK2 (TABLE 2), seem to be promiscuously activated in many types of cancer, and are therefore expected to be good targets for the treatment of many cancers. In haematological malignancies, clinically relevant mutations in signalling proteins are frequently associated with increases in signal transducer and activator of transcription (STAT) 5 activity (BOX 2). Flow cytometry offers the ability to study the signalling activity of such nodes directly, in primary cells, without knowledge of the genetic status of the cells. If a clonal subpopulation with a known mutation arises in a tumour, the per-cell, network-level view of signalling would allow the signalling maps of the wild-type and mutant cells in a sample to be compared.

Signalling in individual cells

Single-cell analysis by flow cytometry is commonly used in the field of immunology, in which specific markers have been used to map *in vivo* phenotypes of tens to hundreds of cell types throughout haematopoietic differentiation. For example, phenotypically distinct B-lineage subsets have been characterized at several steps throughout development from a haematopoietic stem cell to a mature B cell[13]. The role of signalling in

Table 2 | **Frequently altered signalling pathways and their role in cancer**

Cancer cell signalling alteration			References
Ligands and receptors	**Intracellular molecules**	**Acquired capability[§]**	
↑KIT, ↑PDGFR, ↑FLT3, ↑↓BCR, ↑↓TGFβ, ↑IGF1, ↑EGFR, ↑ERBB2	↑SFKs, ↑STAT5, ↑STAT3, ↓NF1, ↑Ras, ↑Raf, ↑ERK, ↑ZAP70, ↑MYC, ↑Smads, ↑PI3K, ↑AKT, ↑SHH, ↑GLI1	Self sufficiency in proliferation	67–85
↓Tumour-necrosis factor family*, ↑decoy receptor family, ↓interferon family[‡]	↓IκB, ↓NF-κB, ↑AKT, ↓p53, ↓caspases, ↓STAT1, ↑BCL2	Evasion of apoptosis, and evasion of killing by the immune system	20,78,79, 86–89
↑αvβ3 integrin, ↑β1 integrins, ↑EGFR, ↑WNT1, ↓E-cadherin	↑SFKs, ↑Ras, ↑Raf, ↑Erk, ↑Rho GTPases, ↑β-catenin, ↓APC	Tissue invasion and metastasis	74,75,77, 88,90–92
↑↓TGFβ, ↓interferon family[‡]	↓ATM, ↓p53, ↓PTEN, ↓RB, ↓STAT1	Insensitivity to anti-proliferative cues	20,71, 77–79,84, 88,93
↑VEGF, ↑VEGFR1, ↑FGF, ↑αvβ3 integrin	↑Ras, ↑Raf, ↑Erk, ↑SFKs	Sustained angiogenesis	74,75,77,81, 90
↑IGF1	↑AKT	Limitless replicative potential	94

*For example, Fas ligand, Fas/CD95, tumour-necrosis-factor-related apoptosis-inducing ligand, and B-lymphocyte-activating factor. [‡]For example, interferon (IFN) receptor α2c, IFNα and IFNγ. [§]See REF. 9.
↑ Indicates that greater than normal signalling activity (through various mechanisms) supports cancer cells.
↓ Indicates that loss of signalling activity supports cancer cells. ↑↓ Indicates that increased or decreased signalling supports cancer cells, depending on the cell type and context. Ligands and receptors: BCR, B-cell receptor; EGFR, epidermal growth factor receptor; FGF, fibroblast growth factor; FLT3, Fms-like tyrosine kinase 3; IGF1, insulin-like growth factor 1; PDGFR, platelet-derived-growth-factor receptor; TGFβ, transforming growth factor β ; VEGF, vascular endothelial growth factor; VEGFR1, VEGF receptor 1. Intracellular molecules: APC, adenomatosis polyposis coli; caspases, cysteine aspartases; ERK, extracellular-regulated kinase; NF1, neurofibromin 1; NF-κB, nuclear factor-κB; PI3K, phosphatidylinositol 3-kinase; PTEN, phosphatase and tensin homologue; RB, retinoblastoma protein; SFKs, Src family kinases (for example, SRC, ABL, LCK and LYN); SHH, sonic hedgehog; ZAP70, ζ-chain-associated protein kinase 70.

Box 2 | **Signalling network effects of clinically relevant genetic changes**

Fms-related tyrosine kinase 3 mutations
- Observed in acute myeloid leukaemia (AML) cells, and is one of the best indicators of poor clinical outcome[51]
- Result in potentiated signal transducer and activator of transcription (STAT) 5 and extracellular-regulated kinase 1 (ERK1)/ERK2 signalling responses in primary AML cells[4]
- Different fms-related tyrosine kinase 3 (FLT3) mutations might each have a distinct signalling profile

KIT mutations
- Observed in gastrointestinal stromal tumours (GIST) and chronic myeloid leukaemia (CML) cells, and are associated with poor clinical outcome in patients with GIST[52]
- Signalling network effects are unknown in primary cells, but STAT5, ERK1/ERK2, and Src family kinase (SFK) signalling are implicated, based on studies in cultured cells

KRAS-, NRAS- and BRAF-activating mutations
- NRAS and BRAF mutations are observed in melanoma and many other cancer cell types[53], and are associated with poor clinical outcome in patients with melanoma[54]
- KRAS mutations are associated with outcome in lung adenocarcinoma[55]
- ERK1/ERK2 signalling are constitutively activated[54,56], but not in all cancer cell types[57]
- A network-level view of these signalling alterations might show overall similarities and differences in the effects of these mutations

Expression of epidermal growth factor receptor and ERBB2
- Observed in breast carcinomas and associated with clinical outcome[58], especially in patients treated with trastuzumab[59]
- Activated AKT and Ras, Raf or ERK1/ERK2 signalling observed in cell lines[60,61]
- Network profiling might indicate potential resistance to therapy and determine whether a drug is capable of suppressing the activated-ERBB2-associated signalling profile

Vascular endothelial growth factor overexpression
- Associated with poor outcome in patients with haematological malignancies[27]
- The vascular endothelial growth factor signalling network, which involves Ras–mitogen-activated protein kinase (MAPK) and SFKs, might be altered in cancer cells

BCR–ABL fusion protein
- Fusion protein that is present in nearly all CML cells
- Inhibited by imatinib, which is effective in treating patients with CML[11]
- Constitutive activation of STAT5 signalling frequently observed[62,63]
- Mutation in ABL region of BCR–ABL arises following therapy with an ABL inhibitor[64,65]
- BCR–ABL signalling-network profile might become re-activated in drug-resistant cells

Mutations in *STAT3* and *STAT5*
- Observed in AML cells[66] and associated with poor outcome in patients
- Changes in STAT3 signalling might affect other signalling nodes in the network
- All activating mutations in *STAT5* might share a common signalling-network profile that could be used to identify cells with mutations in this pathway

development is commonly studied in animal models by deletion or mutation of signalling genes. Such approaches have revealed much about the requirement for signalling in normal differentiation and indicated that differential activation of the same signalling pathways can determine outcomes as distinct as survival and death[14]. However, little is known about the actual timing and magnitude of signalling in cells that undergo these contrasting lineage choices. What differing gene-expression patterns result from a short spike in kinase activity versus a sustained low level of kinase activity? Even less is known about the level of signalling activity that results in crosstalk — a situation in which altered activity of one area of a signalling network alters the outcome of signalling in other areas. The mapping of signalling networks in primary cells through flow cytometry has begun to address these topics, as multiple nodes can now be measured simultaneously in each cell.

Flow cytometry also allows for detection of lineage and oncogene expression, and provides a way to compare tumours that are composed of multiple subclones (FIG. 1). The presence of multiple cell types in a patient sample is an advantage in flow cytometric analysis because each cell population provides a control for the other cell types. The ability to perform this type of internal comparison is especially useful if the cancer cells and their suspected normal precursors are present in the same sample. Signalling maps of different types of normal primary cells can also be compared by combining the ability of flow cytometry to perform both lineage tracking and analysis of cell signalling networks (FIG. 1). For example, the use of lineage markers allows the resolution of two cell populations, and then the signalling status of four node states can be compared for each population. Another advantage of flow cytometry is that the activity of endo-genous signalling proteins can be measured in primary human cells. With this technique, we have observed that activation and deactivation of signalling proteins in both normal and primary cancer cells is more tightly regulated than that in tissue-culture-adapted cell lines of the same lineage (REF. 4) (J.M.I., G.P.N. and R. Levy, unpublished observations).

Signalling stimuli and inhibitors

Important mechanistic information about the signalling network in cancer cells can be determined by treating cells with various stimuli or inhibitors and studying the outcomes. For example, by inhibiting the activity of a specific signalling node with a drug, such as a kinase inhibitor, it is possible to determine if that node is required for the activity of other network nodes[15]. Alternatively, if a node is constitutively activated — for example, through mutations in oncogenes such as fms-related tyrosine kinase 3 (*FLT3*) or *KRAS* (BOX 2) — it can be determined if a node is sufficient to activate other network nodes[4]. Alterations in protein expression levels, such as through overexpression or knockdown, before mapping the signalling network can also be used to study the effect of signalling perturbations.

For the study of cancer, the map of a signalling network that occurs in cells following stimulus (such as with growth factors) plus treatment with a therapeutic agent could be compared with the map that results from treating cells with the stimulus alone. Alterations in signalling profiles of cells following exposure to a therapeutic agent could indicate which signalling nodes are affected by the drug. A significant caveat associated with studies that involve signalling inhibitors is that although they might inactivate a particular signalling node, they can also affect other nodes in the network.

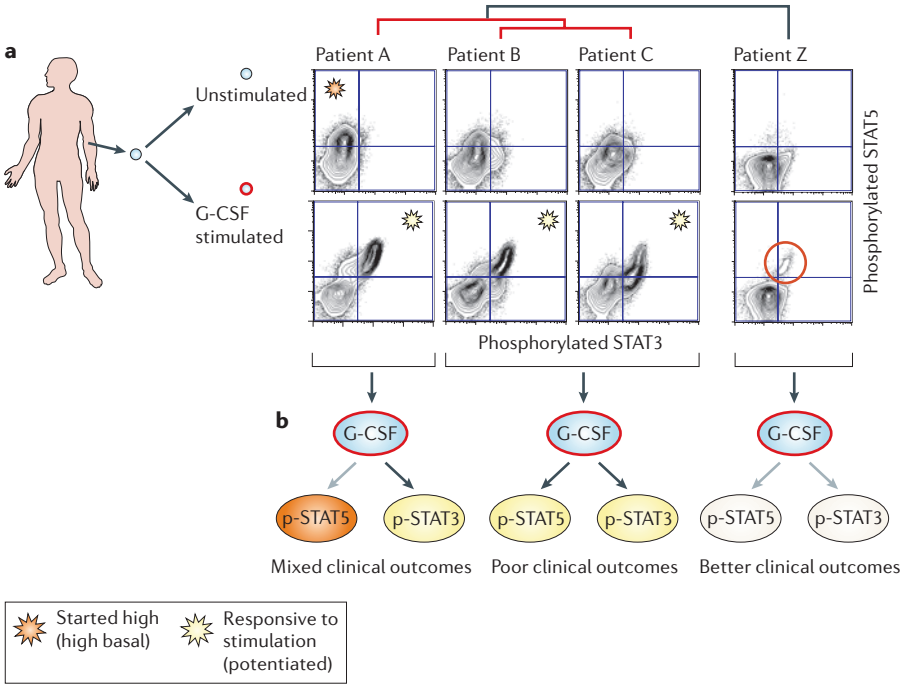

Figure 2 | **Commonality of mechanism indicates a signalling profile. a** | The existence of cell subsets with similar signalling profiles can be used to group patients. Cancer cells obtained at diagnosis from patients A, B and C show similar responses to cytokine stimulation with granulocyte colony-stimulating factor (G-CSF). According to the contour plots, treatment with G-CSF causes more than half of the cancer cells to increase their levels of signal transducer and activator of transcription (STAT) 3 phosphorylation (*x* axis) and STAT5 phosphorylation (*y* axis) in these three patients. However, patient A has a higher basal level of STAT5 signalling than patients B or C, and is therefore distinct. By contrast, most cancer cells from patient Z do not respond to G-CSF stimulation by phosphorylating STAT3 or STAT5. **b** | Network maps are drawn to represent the signalling mechanisms active in each group. Each map can be associated with a different clinical outcome. In this example, the presence of cells with potentiated responses of STAT5 and STAT3 to G-CSF stimulation is associated with an aggressive cancer cell phenotype (middle, patients B and C). The small subset of cells in the sample from patient Z that responds to G-CSF stimulation by phosphorylating STAT5 (red outline in the lower right histogram) indicates that a subpopulation with an aggressive phenotype was present at diagnosis. If patient Z relapses following therapy, we might expect that the post-relapse signalling profile would appear more like that of patient B at the time of diagnosis.

For example, farnesyl-transferase inhibitors are reported to have significant additional effects on cell signalling[16], and the classic 'targeted' inhibitor imatinib has activity against several mutant kinases, including KIT, platelet-derived-growth-factor receptor-β (PDGFRβ) and ABL[17,18]. With a network-level view of signalling in cells with and without an inhibitor present, the mechanism of drug action can be measured directly by measuring key signalling nodes throughout a cell. Furthermore, a drug's effect in both tumour and non-tumour cells within a sample can be distinguished by flow cytometric analysis.

A minimal starting point in the choice of signalling inputs is to find an activator of every signalling node that will be analysed. Cells that are treated with the stimulus are used as positive controls for signalling activity, whereas cells in the basal state (resting cells) function as the negative controls. However, because some signalling pathways are constitutively activated in some cell types, it might also be necessary to suppress certain signalling nodes using inhibitors.

Measuring active signalling

To understand changes in the regulation of signalling it is important to determine not only whether a particular signalling pathway is constitutively active (high-level basal signalling), but also to determine how signalling responses differ in cancer cells. Such differences are commonly observed in normal cells, which display significant heterogeneity in signalling responses owing to lineage-specific expression of signalling network molecules and their regulators. An example of this can be seen in the differences in the phosphorylation of STAT6, in response to interleukin (IL)-7 treatment, between B and T cells (FIG. 1). Differences in the quality of signalling (magnitude and duration) are also observed in normal cells and are expected to be seen in cancer cells. For example, all mature B cells express a B-cell receptor and have the ability to phosphorylate ERK1/ERK2 in response to B-cell receptor ligation. However, B cells of different B-cell receptor heavy-chain isotypes (for example, immunoglobulin (Ig) M versus IgG) differ in the kinetics of ERK1/ERK2 activation and inactivation (J.M.I., G.P.N. and R. Levy, unpublished observations). So, sustained ERK1/ERK2 phosphorylation following B-cell receptor stimulation is a signalling response that distinguishes B-cell isotypes. In both of these examples, a potentially important difference in the activity of two signalling networks cannot be measured by observing basal signalling alone.

A clinically relevant signalling profile might require other information that can only be obtained by measuring the responsiveness of signalling network nodes in live cells. For example, the loss of key anticancer signalling nodes is common in oncogenesis (TABLE 2), and failure to signal cannot be assessed without providing a signalling input. One example is the loss of normal STAT1 signalling in response to interferon-γ (IFNγ) treatment, which has been observed in AML[4] and other cancer cell types[19]. This type of Janus kinase (JAK)–STAT signalling revision is depicted in FIG. 2. The IFNγ-mediated activation of STAT1 transcriptional activity is an important signalling event in cancer (TABLE 2) because activation of STAT1 regulates key cell activities, including display of antigens to the immune system, cell-cycle arrest and p53 activity[19–21]. The chain of signalling events between IFNγ stimulation and STAT1 phosphorylation might be compromised in various ways in different cancer cells, but the resulting altered signalling mechanism is the same, and this aberrant signalling mechanism — failure of IFNγ to activate STAT1 phosphorylation — is not apparent in a resting cancer cell.

Features of cancer cell signalling networks

Comparing signalling networks among patients can be used to identify signalling pathways associated with gene mutation (BOX 2) and to relate signalling profiles with clinical outcome[4] (FIG. 3). In this way, measuring signalling networks in single cells can lead to insights about mechanism and cell type. Phosphorylation of ERK1/ERK2

leads to cell proliferation, and increased ERK1/ERK2 signalling is a common feature of cancer cells. In cases in which increased ERK1/ERK2 signalling is observed in aggressive cancer cells, compared with those that respond to therapy, ERK1/ERK2 phosphorylation will therefore be a useful biomarker for risk stratification and, potentially, for determining therapy. Increased phosphorylation of ERK1/ERK2 in response to FLT3-ligand stimulation was one part of a profile that has been associated with resistance of AML blast cells to the first course of chemotherapy (with idarubicin and cytarabine)[4].

Signalling alterations that commonly occur in cancer cells often result in survival and proliferation, decreased activation of anti-proliferative signals, and altered responses to external stimuli. All three of these phenotypes were observed in cells from patients with AML, and studies of these cells have led to insights into mechanisms of pathogenesis and clinical response. For example, increased activity of STAT5 and STAT3 — transcription factors that induce expression of genes that enable cell survival and proliferation (FIG. 2) — were observed in AML blast cells following stimulation with cytokines such as granulocyte colony-stimulating factor (G-CSF) and IL-3. Additionally, cells from some patients with AML failed to phosphorylate STAT1 in response to IFNγ. Phosphorylation of STAT1 is a cytokine signalling response that is normally anti-proliferative. Other patient samples showed alterations, such as activation of STAT5 instead of STAT1, in the response to IFNγ. By grouping samples according to signalling profile and associating each with clinical outcome, certain altered signalling patterns could be characterized as 'aggressive' and associated with poor response to chemotherapy (FIG. 3).

A key observation from measuring these alterations was that cancer cells from patients with poor clinical outcome showed all three phenotypes (activated pro-proliferative signalling pathways, inactivated anti-proliferative signalling, and not responding properly to external stimuli), whereas cancer cells from patients with better clinical outcomes only had one or two of these features. So, the signalling profile associated with the most aggressive cancer cells was one that conferred just enough aggressive signalling behaviour to support cancer cell survival and proliferation without triggering arrest, cell death, or detection by the immune system.

Challenges in clinical application

Developing the use of multi-parameter fluoresence-activated cell signalling (FACS) for the identification of signalling profiles of cancer cells from patient samples offers many opportunities and faces many challenges (BOX 3). For example, just because a particular signalling network is activated in a cancer cell type, this does not mean that it is required for cancer progression, or that targeting the network will improve a patient's outcome. Signalling profiles might only be biomarkers that are associated with clinical outcome, and not mechanisms that are required for continued survival, proliferation and resistance of cancer cells to therapy. To resolve these possibilities, signalling inhibitors that block the features of aggressive signalling

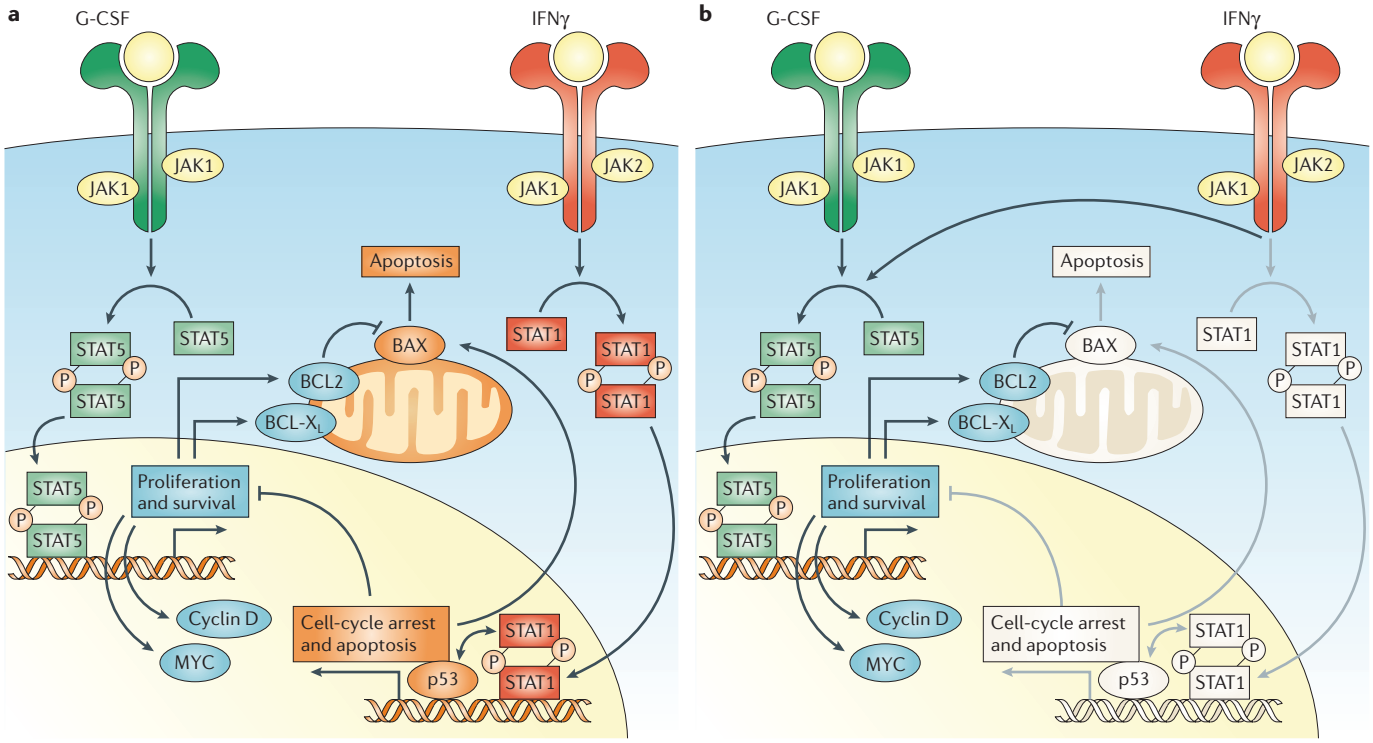

Figure 3 | **Changes in JAK–STAT signalling in therapy-resistant cancer cells.** Signalling profiles of two different cancer cells are shown. **a** | In this cancer cell, granulocyte colony-stimulating factor (G-CSF) signalling through Janus kinase 1 (JAK1) results in signal transducer and activator of transcription (STAT) 5 phosphorylation (P), nuclear translocation and activation of genes that mediate proliferation and survival. At the same time, interferon-γ (IFNγ) signalling through JAK2 results in STAT1 phosphorylation, nuclear translocation, and transcription of genes that mediate cell-cycle arrest and apoptosis. As a result, DNA-damaging therapy might still be effective for patients whose cells show this profile. **b** | In the signalling network of the second cancer cell, the IFNγ signalling has been rerouted to activate STAT5, which results in transcription of pro-survival and proliferation genes, as opposed to the anti-proliferative effects of STAT1. Patients whose cancer cells show this signalling profile might be more resistant to DNA-damage-induction therapy. Inhibition of STAT5, the focal node activated in the therapy-resistant cancer cell, might therefore shift the balance between proliferation and apoptosis, and improve the response to cancer therapy.

profiles must be tested in animal models and human clinical trials, to distinguish between markers and mechanisms.

It is also not clear how many of the altered nodes in a cancer signalling network would need to be modulated by an anti-cancer therapy. It is possible that removing just one key signalling element, such as STAT5 activation, would be sufficient to turn a therapy-resistant cancer cell into a therapy-responsive cell. It also remains to be determined whether therapies that inhibit key signalling network nodes, which are common to many cell types, would be specific to cancer cells or would cause side-effects in non-cancer cells. Flow cytometry is a useful way to approach this question because signalling in normal and cancerous cells can be compared in the same sample (FIG. 1). The clinical success of targeted therapies, such as imatinib, indicate that modulation of signalling could safely improve clinical outcomes for other patients with cancers in which the signalling mechanisms are known[22], but this must be studied on a case-by-case basis for each drug.

What advantages do flow cytometric maps of cell signalling provide over other biomarker detection systems, and what are the challenges to this analysis? Although analysis of signalling networks by flow cytometry takes only a few hours and uses relatively small clinical samples, there are some challenges in adapting this technique for the clinic. For a signalling profiles approach, patient specimens must be viable (at the time of stimulation) to determine altered signalling responses. Additional challenges of clinical application of flow cytometry have been reviewed[23]. Especially important are flow cytometry-compatible biomarkers for cancer cells (TABLE 1) — such as phospho-specific antibodies that can detect epitopes in fixed, permeabilized cells[1,2] — and the number of parameters that can be detected per cell[3].

Another significant issue is that of 'culture shock' — the process by which primary cells change their behaviour to adapt to cell culture. Ideally, primary human cells are studied in *ex vivo* assays that involve minimal manipulation after removal from the body. Protocols for the whole-blood stimulation of lymphocytes before fixation and flow cytometric analysis of signalling have helped to address this challenge[24]. For solid tissues, it is not clear to what extent the dissociation required for flow cytometric analysis would interfere with the useful analysis of signalling network structure.

Box 3 | **Cancer biology challenges that can be addressed by single-cell signalling profiles**

- Pinpoint alterations in single cells and cell subsets. What signalling mechanisms are active in cancer cells that return during patient relapse, in pre-metastatic cells and during the earliest stages of transformation?
- Look not only at 'pathways', but at the network as a whole. What are the 'on target' and 'off target' effects of drugs?
- Identify and track cancer stem cells. Is there a phenotypically distinct subset of cells that is not killed by therapy and that mediates relapse?
- Identify targets for drug discovery. What signalling mechanisms enable cancer cells to resist a particular chemotherapy?
- Choose an optimal therapy. Do patients that respond to a particular cancer therapy have similar signalling profiles?
- Monitor anticancer therapies. Can signalling profiles be used as biomarkers of therapeutic response or side effects?
- Detect cancer earlier. Can signalling profiles of circulating cancer cells, or of immune system cells, be used to detect cancer at early stages?
- Understand mechanisms of cell–cell and cancer-cell–host interactions. How do cancer cells interact with and alter the host microenvironment or immune system?

To date, this technique has been used to study haematopoietic malignancies, including disaggregated lymph-node tumours (J.M.I., G.P.N. and R. Levy, unpublished observations), but has not been applied to other solid tumours. In the case of some solid tumours, analysis of altered signalling in infiltrating immune cells could be a more useful approach than disaggregation and analysis of the cancerous cells themselves.

Currently, data analysis also presents a significant bottleneck. New informatics techniques are necessary for multiple cancer-cell biomarkers to be tracked under various stimulation conditions and to be compared as cell populations among patients. If two biomarkers are compared (FIG. 1), it is easy to portray subset-specific signalling. However, displaying a signalling network map for tens of cell subsets that are present to different degrees across a set of hundreds of patient samples would be difficult during data analysis and extremely challenging to portray in a static figure for publication. Perfetto *et al.* describe automated data-analysis techniques and methods to explore complex, multi-parameter data sets that might be good starting points for the signalling-profiling approach[3]. For data sets that include signalling networks that are specifically perturbed at one node, such as through small-molecule inhibition or mutational activation of a signalling protein, causal analysis using machine-learning methods (for example, Bayesian network analysis) can be used to automatically map the signalling network for each cell in a population[15].

Ultimately, researchers and clinicians might require access to dynamic visualizations of primary data along with experimental interpretation. An infrastructure such as the one developed for the National Center for Biotechnology Information gene-expression omnibus could provide the storage and efficient retrieval of large amounts of data that is required for this effort. Ideally, signalling network maps for cells in a population and signalling profiles for patients in a cohort would all be securely available online. The data would be interconnected to allow study of a group of patients to include visualization of the common signalling profile as well as analysis of the individual cell maps of signalling networks underlying the profile.

These challenges, although significant, are not insurmountable and will be tackled with the adoption of flow cytometry as a tool to study cell signalling.

Future directions

Many new ways of studying cancer biology are possible using individual-cell studies of signalling at the network level. One example is using flow cytometry to track populations of cancer cell subsets from individual patients at different stages of treatment. This would be especially informative in identifying mechanisms of resistance to therapy, and could address a key goal in the field — to identify cells within a heterogeneous tumour sample that should be targeted by therapy (BOX 3). It has been proposed that the tumour microenvironment can alter the signalling of infiltrating host immune cells[25], surrounding stromal cells[26] and the vascular network[27]. A single-cell approach can also

be used to study such changes in host cells that are present in samples from cancer patients (BOX 3).

As the populations of cells in a patient change over the course of anticancer treatment, the associated changes in the signalling network maps of tumour cells can be monitored and the overall signalling profile of the patient can be updated. Furthermore, by tracking different subsets of cancer cells, it might be possible to identify and study groups of therapy-resistant cells to enrich our understanding of immunoediting[28] — signalling alterations that allow cell populations to evade immune detection (BOX 3; FIG. 3). The ability to monitor multiple samples from the same patient over time also make it possible to characterize transformed cells or pre-transformation cells in samples that were obtained at the time of diagnosis and compare them with samples taken from later-stage disease (BOX 3).

A comparison of signalling profiles of different cancer types might reveal common features of aggressive cancer cells. For example, it will be interesting to compare alterations in the signalling networks of various haematological malignancies, such as AML, CML, acute lymphoblastic leukaemia, chronic lymphocytic leukaemia, juvenile myelomonocytic leukaemia, multiple myeloma, follicular lymphoma and diffuse large B-cell lymphoma. Cell types of haematopoietic origin should share common signalling components, and these malignancies would presumably be less-distantly related to each other than they would be to solid tumours. These types of comparisons might identify signalling network alterations that are commonly associated with poor clinical outcome. Other features of cancer progression not discussed in detail here, such as induction of angiogenesis or metastasis, might also be driven by common signalling alterations in different cancer types and could be studied using flow cytometry.

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Competing interests statement
The authors declare competing financial interests: see web version for details.

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