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A Systems Perspective of Heterocellular Signaling

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Abstract

Signal exchange between different cell types is essential for development and function of multicellular organisms, and its dysregulation is causal in many diseases. Unfortunately, most cell-signaling work has employed single cell types grown under conditions unrelated to their native context. Recent technical developments have started to provide the tools needed to follow signaling between multiple cell types, but gaps in the information they provide have limited their usefulness in building realistic models of heterocellular signaling. Currently, only targeted assays have the necessary sensitivity, selectivity and spatial resolution to usefully probe heterocellular signaling processes, but these are best used to test specific, mechanistic models. Decades of systems biology research with monocultures has provided a solid foundation for building models of heterocellular signaling, but current models lack a realistic description of regulated proteolysis and the feedback processes triggered within and between cells. Identification and understanding of key regulatory processes in the extracellular environment and of recursive signaling patterns between cells will be essential to building predictive models of heterocellular systems.

Introduction

A primary driver of systems biology is to understand how biological components are organized into networks and to discern the patterns of these interactions giving rise to emergent properties (1). Most systems biology studies have focused on how intracellular protein networks control cell metabolism and behavior. Protein networks, however, are only one level of a complex regulatory hierarchy by which organisms respond to their environment. The next higher level involves the networks that dictate how cells organize to form tissues, and how perturbations of these lead to pathologies.

Biochemically based regulatory networks control how cells organize into tissues and how tissue function is coordinated to maintain physiological homeostasis (e.g. (2)).

Understanding the basis of these networks is fundamental to understanding human health and disease. Indeed, delineating the hormonal network controlling human physiology was

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one of the greatest achievements of 20th century biology, and resulted in successful treatments of previously lethal diseases (3). These relatively well-understood endocrine and exocrine networks coordinate tissue function, but many additional regulatory networks coordinate cell functions within tissues.

The networks of growth factors and regulators controlling cell-to-cell communication in tissues are far less understood than the higher-level endocrine/exocrine networks and lower level signaling pathways. Nevertheless, they serve a crucial role in fundamental processes such as embryonic development and wound healing (4, 5). Their disruption can lead to pathologies that include cancer, dysplasias and chronic wounds and scarring (6). Despite the importance of these heterocellular signaling networks, they are studied infrequently, mostly because of technical difficulties and their inherent complexity. Advances in analytical technologies and improved understanding of biological networks have begun removing some of the barriers to investigating heterocellular networks (7) and promise to provide new approaches to understand and treat many chronic diseases arising from their dysregulation.

Limits of current technologies and approaches

Biological research into cell behavior has traditionally focused on either individual cell types or whole tissues, but rarely used heterocellular systems because of difficulties in working on them. Tissues can be reliably studied in situ or in organ culture (8). Monocultures also can be very reproducible with respect to growth, density and physiological function. When multiple cell types are combined, however, this tidiness disappears. Cells will compete for available nutrients and secrete factors that can enhance or inhibit the growth of their neighbors (9, 10). Unless extreme care is taken to manage the density, position and microenvironment of mixed-cell cultures, it is very difficult to achieve consistent, reproducible results that can be more easily interpreted.

Recent advances in engineering artificial substrates and microenvironments for mixed-cell cultures promise to alleviate some technical issues. These “organs on a chip” also provide a way to investigate the contribution of spatial and mechanical aspects to cell functions (11, 12). Currently, they constitute a challenging and highly specialized area of research, but continuing advances promise to make these systems mainstream over the next decade. Meanwhile, investigating mixed cell systems is primarily restricted to in situ animal studies of either normal tissues or reconstituted tissues (e.g., human skin models (13)).

Rather than analyzing a mixture of cells as a single unit, one can separate cell population following an experimental treatment to individually analyze marker-tagged cells using a spectrum of specific antibodies or RNA-Seq (14, 15). The introduction of rare-earth metal-based antibodies in conjunction with specialized flow cytometers has greatly expanded the number of antibodies that can be used simultaneously in single-cell studies (16, 17), but this approach is intrinsically limited by the need for specific, high-avidity antibodies. It also requires prior knowledge of the most important targets. Separating cells before analysis also results in loss of information regarding the spatial context regulating many heterocellular signaling systems, and separation itself can alter the signaling networks.

Heterocellular signaling can also be monitored in situ using fluorescent or rare-earth metal-labeled antibodies (18). This preserves spatial information, but again requires specific antibodies and specialized instrumentation and preparation techniques. In situ RNA-Seq approaches have also been developed (FISSEQ; (19)) to analyze transcriptional response profiles at the single-cell level. However, these techniques produce very complex datasets that are difficult to analyze (20). Protein-based assays usually provide semi-quantitative information on protein abundance and modification state, but not cell physiology (21). Single-cell transcriptomics can provide information on cell responses but inferring the cell's protein-based signaling network from them is unreliable (Shi ref) and does include post-translation modifications and processing that regulate protein activities (20).

Systems perspective of heterocellular signaling

Fundamentally, productive research on any system, especially heterocellular signaling, requires a prior expectation of how it works. Fortunately, decades of research on monocultures provide a solid foundation on which to base heterocellular hypotheses. For monocultures, the experimentalist typically provides the signals that would normally come from neighboring cells, such as soluble growth factors, extracellular matrix and nutrients. What such systems lack, however, is the spatial heterogeneity of normal intercellular signaling and the complex feedback processes triggered when cells are perturbed. This lack of appropriate cellular context limits the ability to predict how heterocellular systems will respond to a stimulus.

Growth factors and cytokines (i.e., ligands) are produced locally in natural heterocellular systems and encode information regarding the extracellular environment. These are the “cues” in the “cue-signal-response” paradigm (22). However, the typical signal transduction experiment uses a fixed bolus of ligand containing only two pieces of information: the identity and concentration of the ligand. In situ, the spatial location of the ligand and its production rate are dynamic (23, 24). It has been shown that receptor binding and signaling is spatially restricted, such as in polarized cells, due to non-homogenous distribution of receptors and their substrates (25). Signaling is also impacted by ligand fluctuations from a heterogeneous microenvironment, clearance and lateral crosstalk (26). Thus, typical experiments do not provide growth factor signals in a physiologically relevant way.

Understanding how the spatial distribution and dynamics of ligands is controlled is crucial for understanding heterocellular signaling because any initial pattern will be either reinforced or modified by responding cells that produce additional signals and growth factors (27). One of the most important mechanisms for this feedback is activation of specific proteases, such as ADAM 17, which process and release growth factor precursors at the cell surface (28). Increased expression of growth factors and cytokine genes are also frequently seen following cell stimulation, suggesting a complex cascade of altered ligand production (29). Thus, at the tissue level, growth factor signaling is a context-dependent conversation between cells rather than a singular response.

As an example, consider the skin—a stratified tissue with clear spatial delineation between the dermis and epidermis cells. Under normal conditions, the skin is at homeostasis, with the

basal keratinocytes proliferating and then vertically differentiating with subsequent enucleation to form a keratinized layer. This process involves not just cell-intrinsic differentiation programs but also processing of extracellular substrates (30). During this epidermal activity, the dermis remains relatively quiescent with little proliferation or matrix production by the resident fibroblasts, and a stable but highly limited vascular network. It is thought that these two states of consistent epidermal cell replacement and quiescent dermal activities is communicated by signaling between the compartments, though these networks are ill defined.

However, when perturbed, both the epidermal and dermal compartments respond in a linked manner that implicates a communicating network; e.g., the response to mechanical pressure that leads to calluses (31). In this situation, there is combined hypertrophy of both layers with a commensurate increase in thickness and cell numbers. In addition, the matrix, which forms the bulk of the dermal volume, also is increased. It remains to be determined whether the signals are simply mechanical stimuli transmitted to all the affected cells or, more likely, a biochemical feedback network invoked by the mechanical forces.

A more dramatic heterocellular signaling response occurs during excisional wound healing, with its progression through stages that are characterized by distinct signaling networks and cell responses (Fig 1) (32, 33). The initial loss of tissue and severing of the vascular conduits leads to bleeding and a hemostatic plug that includes activated platelets and trapped immune cells. The clotting cascade activates numerous proteases and generates bio-active peptides while platelets release signaling peptides and small metabolites (e.g. HB-EGF, PDGF, PF4 and VEGF), and the neutrophils secrete many cyto- and chemo-kines. These factors induce cellular dedifferentiation with the basal and suprabasal keratinocytes undergoing a temporary epithelial-mesenchymal transition to proliferate and migrate into the provisional matrix of the clot. The fibroblasts and endothelial cells undergo mitogenesis at the edges before migrating into the spaces to replace the lost tissue. The early, exuberant tissue replacement is relatively uncoordinated with cells appearing in inappropriate spaces (e.g., keratinocytes in the deep dermis), but as the matrix matures, a basement membrane is reestablished, and the cells revert to a more differentiated state. Signaling networks drive this tissue regeneration as evidenced by a more rapid wound closure following augmentation with specific growth factors (34, 35).

Interestingly, the role of signaling networks is most evident during the latter, resolving phases of wound healing. There is an imperative to close the wound to prevent infection and water loss that usually results in excessive scarring. During the resolving phase of healing, however, ~50% of the epidermal cells and fibroblasts are lost, and up to 90% of the new blood vessels involute (36). This dramatic pruning is under the control of signals turned on as the wound cells “sense” closure. Chief among these networks is signaling through the CXCR3 receptor since the absence of this receptor leads to hypertrophic scarring and excessive vascularity (37). Conversely, analysis of non-healed human wounds shows suggests that excessive protease activities can create a wound bed inhospitable to tissue regeneration (34, 38).

Mechanisms of heterocellular signaling

The complex sequence of events in wound healing clearly depends on intercellular signaling networks, but the identity of the most important ones and how they are sequentially regulated is poorly understood. Although studies of cell monocultures have provided insights regarding basic signaling mechanisms, heterocellular signaling studies need to be extended to include the spatial-temporal aspects of growth factor generation, receptor access and substrate availability.

The spatial modes of growth factor signaling are traditionally classified as paracrine, autocrine and juxtacrine (Fig. 2) (39). Each mode of signaling is dependent on the physical accessibility of both ligands and their receptors and thus intrinsically contains information regarding different spatial-chemical aspects of the extracellular environment. Some ligand-receptor systems are specialized for one mode of signaling, such as the ephrin and cadherin systems being specialized for juxtacrine (40, 41). Other systems can operate in multiple modes, notably the EGFR system, which can operate in all three (39). In this case, it has been shown that each signaling mode can cause distinct biological responses, implying that cells can decode information from spatially restricted signaling. This is consistent with studies showing that restricting the location of ligand-receptor complexes can qualitatively change cell signaling. For example, when activated EGFR are restricted to the apical surface of polarized epithelial cells, only receptor substrates co-localized to the apical surface are phosphorylated (25). Juxtacrine signaling is the most spatially restricted process, requiring direct cell-cell contact. Autocrine signaling will be mostly restricted to sites near ligand release. Paracrine signaling by definition operates at the multicellular level, conveying information between different cell types. How these signaling modes are differentially regulated provides insight into how cells monitor and interpret their extracellular environment.

Juxtacrine

Juxtacrine signaling is defined by a ligand and receptor on adjacent cells forming a stable signaling complex. In specialized juxtacrine systems, such as the ephrin-Eph receptor system, ligands can only activate their cognate receptors when attached to a membrane due to the need for ligand clustering (42). Such physical restrictions are central to the role that the Eph receptor system plays in axon guidance, angiogenesis and epithelial cell migration (40). In other juxtacrine systems, signaling occurs only when the appropriate ligand and accessory molecules are expressed together on the cell surface (e.g., membrane-bound amphiregulin and CD9 to activate the EGFR (43)).

Membrane-associated proteases, notably members of the ADAMs family of metalloproteases, play a central, though diverse role in regulating juxtacrine signaling. Cleavage of the membrane-associated Ephrins by ADAM10 is thought to terminate receptor signaling by eliminating ligand-mediated receptor clustering and allowing endocytosis and degradation of the released ligand-receptor complex (44). In contrast, cleavage of the Notch receptor by ADAM10 after binding its juxtacrine ligand is the primary activation event in the Notch signaling pathway (45). Interestingly, proteolysis of the EGFR juxtacrine ligand HB-

EGF by ADAM17 converts it from a juxtacrine to an autocrine factor with a consequent change in its biological activity (46). As we will see below, regulated proteolysis is a major regulator of heterocellular signaling, but its impact is highly dependent on the specific ligand-receptor pair.

Autocrine

Autocrine signaling is when a cell simultaneously produces a soluble ligand and the receptor to which it binds. This simple definition belies the complexity of most autocrine systems because the ability of released ligands to bind to their receptor depends on many different physiochemical parameters, such as surface receptor density and ligand affinity (47, 48). The strong contextual dependency of autocrine signaling provides many potential levels of regulatory control, which is likely responsible for its high prevalence in multicellular organisms. Transcriptome analysis of 144 primary human cell types indicates that most express tens to hundreds of different receptors and ligands, ~66% of which are potentially autocrine systems (49).

Most autocrine ligands must be proteolytically processed before they can bind to their cognate receptor. Some ligands can act as both juxtacrine or soluble ligands, such as HB-EGF, but most seem to require cleavage for activity. For example, the activity of membrane-anchored TGF- α , an EGFR ligand, is directly proportional to the activity of its releasing protease, ADAM17 (50). Thus, as in the case of juxtacrine signaling, autocrine signaling is regulated by proteolytic processing.

One of the most interesting aspects of autocrine signaling is that its spatial range can be regulated at both the receptor and ligand levels. In a high-affinity ligand-receptor system (e.g., TGF- α and the highly expressed EGFR in epithelial cells), ligand capture occurs very close to the release site (47). For lower affinity ligands, such as amphiregulin (AR), or in cells expressing low levels of receptors, ligand capture occurs over much larger distances (48).

Release of autocrine ligands is primarily controlled by other signaling pathways, such as GPCRs (e.g., Angiotensin, LPA) that can stimulate proteases, such as ADAM17, by inducing the phosphorylation of iRhoms (51). Phosphorylation-modulated proteins, such as the iRhoms, seem to function as integrators of multiple kinase signals, such as Src and Erk (52). Autocrine signaling itself can stimulate ligand shedding in a positive feedback loop (27). Thus, the overall activity of shedding proteases reflects the integration of multiple input and feedback signals.

Paracrine

Paracrine signaling, where ligands and receptors are produced by distinct cell types, is prominent in physiological processes where the behavior of different cell types needs to be coordinated, such as angiogenesis and tissue remodeling (39). However, paracrine signaling is often highly dependent on extracellular context, providing opportunities for fine-level regulatory control. For example, hepatocyte growth factor (HGF) produced by fibroblasts is synthesized as an inactive, secreted precursor that must be cleaved by the protease

hepatocyte growth factor activator (HGFA). HGFA, in turn, is activated downstream of the blood coagulation cascade (53). Thus, net HGF activity depends on both regulated synthesis and an appropriate extracellular context.

In the case of insulin-like growth factor (IGF-1), a variety of different binding proteins controls its availability and activity (54). The activities of these binding proteins in turn are controlled by proteolytic processing and interaction with proteoglycans (54). Thus, net IGF-1 availability is a complex function of synthesis, release, processing and extracellular context. Understanding how cells both generate and decode the complex pattern of extracellular context that control growth factor activities is one of the greatest challenges in understanding heterocellular signaling.

Matrix-mediated communication

Another prominent regulator of heterocellular context is the extracellular matrix. The matrix can behave as a growth factor by activating matrix-specific receptors, such as the integrins. Some matrix molecules, such as tenascin-C or laminin V, can also activate the EGFR through its EGF-like repeats (55, 56). Ligands presented in this type of 'matricrine' signaling are physically constrained and display affinities significantly lower than cognate soluble autocrine and paracrine ligands (57). Despite the numerous intrinsic receptor-binding sites (including those for integrins and discoidin domain receptors (58, 59)), the matrix appears to have its greatest impact as a physical structure to spatially constrain cellular organization and as a "programmable" scaffold for the sequential promotion of growth factor activation and binding to their receptors (e.g. (60)). This is particularly relevant in the case of excisional wounds where the matrix and the adherent cell change dynamically during the healing process.

The extracellular matrix itself is synthesized by the combined activity of multiple cell types, such as epithelial cells and fibroblasts with each contributing specific protein components (61). Matrix proteins, such as collagen and fibronectin, engage different sets of cell surface integrins to facilitate cellular attachment, migration and organization (9). These processes are also impacted by matrix stiffness and architecture, which also provide structural support for tissues (62). The relative insolubility of the matrix has hindered biochemical investigations of its components and their impact on cell functions, but new technical approaches are making headway with this problem (63).

Many studies have shown that highly charged glycoproteins and proteoglycans, which are abundant components of the matrix interact with a significant number of cell-released growth factors (e.g., HB-EGF, AR, FGF-2) (9). These interactions not only slow the effective diffusion of growth factors but facilitate the activity of many of them. In addition, the ability of cells to respond to growth factors is frequently dependent on their attachment to specific matrix components, such as laminin (64). IGF-binding proteins (IGFBPs) can associate with the matrix and control the availability and activity of IGF-1 (65). Thus, the matrix exerts a profound influence on heterocellular signaling. Conversely, growth factors such as TGF- β can strongly stimulate the synthesis of matrix components (66). This bi-

directional signaling between multiple cell types and the matrix is a crucial level of feedback during tissue organization and development.

Regulation of heterocellular signaling by proteases

Most growth factor signaling is ultimately controlled by regulated proteolysis. The release of tumor necrosis factor (TNF) and the EGF family of ligands, these proteases are regulated by phosphorylation of the iRhoms, which in turn is controlled by multiple signaling pathways (52). Regulated proteolysis can also shut off signaling by releasing the extracellular ligand-binding domain of receptors, such as AXL (67). There are some indications that the released, intracellular domain of cleaved receptors can also signal (68).

Synthesis and release of matrix metalloproteases (MMPs) can facilitate cell responses, such as migration, and positively affect heterocellular signaling by releasing growth factors and cytokines bound to the matrix or cell surface (69). They can also negatively regulate signaling by inactivating certain cytokines. In turn, the activity of MMPs is frequently regulated by extracellular proteases (70). Thus, the MMPs are part of a complex regulatory web of proteases and protease inhibitors that control the dynamics of extracellular growth factor and cytokine activity.

One of the challenges to understanding all of the roles played by proteases in heterocellular signaling is the difficulty in identifying their physiological substrates because of the broader specificity of proteases as compared to growth factor-cytokine receptor binding (70). This makes them ideal regulators for a wide range of different cellular and extracellular activities but creates technical challenges in assigning specific proteases to specific regulatory events. Recent advances in proteomics approaches for studying the “substrate degradome” of proteases promises to clarify some of these uncertainties (71).

The future of heterocellular signaling

A systems-level understanding of heterocellular signaling will require building computational models that can predict the impact of different perturbations. Building and parameterizing such models, however, will require identification and measurement of the key processes controlling heterocellular interactions. To date, most studies of heterocellular signaling have focused on signaling processes within individual cells, but these are strongly impacted by extracellular regulatory processes. In addition, identifying active signaling pathways doesn't necessarily indicate which ligands or receptors are responsible. Multiple ligands can activate a given receptor, and there is considerable overlap and crosstalk between different receptor pathways (72). Ligands also tend to be rapidly consumed following proteolytic release, and thus are very rarely detected, even using deep, ultrasensitive proteomic assays (73). Consequently, most current technologies provide only a fragmented picture of heterocellular signaling insufficient for building systems-level models.

Building more realistic and predictive models will require expanding signaling analyses from intracellular to extracellular events, including those at cell-cell and cell-matrix interfaces (Fig. 3). Even though many of the most important processes are known to be controlled by regulated proteolysis, this is rarely detected by most analytical approaches.

Recent progress has been made in designing artificial matrices and fluorescent substrates that can provide significantly more information on extracellular processes (74). Combining new detection technologies with advanced imaging approaches can start unraveling the spatial-temporal regulatory events driving heterocellular interactions and provide the foundation for systems-level modeling.

Ultimately, understanding heterocellular signaling will require building multiscale models that include both molecular and tissue scales. Such models have been built to describe how the regulated activities of cardiac cell ion channels cause electrical patterns of the heart (5), but these models were based on a firm understanding of the physics of electrical conductivity of tissues and the biochemistry of ion channels. This level of understanding is lacking for regulated proteolysis and cell-matrix interactions. The strong spatial constraints placed on heterocellular signaling also require models with a fine level of spatial granularity, which will place a high computational burden on realistic models. Clearly, new methodologies and computational algorithms for building biochemically accurate multiscale models are needed as well as new approaches for the associated multiscale experiments.

In the meantime, the increased sensitivity and spatial resolution of high-throughput omics analyses will continue to reveal the molecular events occurring during heterocellular signaling. Transcriptomics profiling of different primary cell types has also provided important information on differences in their gene expression pattern (49). Recent advances in proteomics analyses of extremely small tissue samples (e.g., from laser-capture microdissection) provide an opportunity to quantify both the protein and gene expression patterns of tissues at high spatial resolution (75). If combined with imaging-based analyses of extracellular processes, these new technologies can provide the foundation for building predictive heterocellular models.

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Abbreviations:

ADAM17	a disintegrin and metalloprotease domain 17
AR	amphiregulin
CXCR3	C-X-C motif chemokine receptor 3
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor

ERK	extracellular regulated kinase
FGF	fibroblast growth factor
HBEGF	heparin-binding EGF-like growth factor
PDGF	platelet-derived growth factor
PF4	platelet factor 4
VEGF	vascular endothelial growth factor

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Figure 1: Heterocellular signaling in wound healing.

During the three main stages of excisional wound healing both the matrix composition and the cellular behaviors transition to first seal the void, replace the tissue, and then revert to a quiescent homeostatic state. Adapted from (33).

Figure 2: Three primary modes of growth factor signaling.

In autocrine, cells make both the ligand and the cognate receptor with binding typically occurring with the same cell type. In paracrine, ligands and receptors are made in different cells, although the ligand might not be fully active when released. In juxtacrine, the growth factor is membrane anchored and signals to an adjacent cell.

Figure 3: Information from different signaling systems can provide the logic to specify heterocellular responses.

There are multiple context-dependent signals that can activate or inactive signaling. If a shedding protease is active [1] together with a membrane-bound growth factor and the appropriate auxiliary protein is present on a neighboring cell [2], as in the case with CD9 and HB-EGF, or if a juxtacrine complex is formed [3], then transcription can occur [4]. Some proteoglycans and binding proteins on neighboring cells can inhibit signaling [5]. In many cases, cells will require additional signals to activate transcription, such as binding to the extracellular matrix [6] or a permissive growth factor, such as IGF-1 [7].