SPECIAL REPORT

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Cell density regulates cancer metastasis via the Hippo pathway

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Metastatic spread of cancer cells from the primary tumor site to distant organs is the major cause of death in cancer patients. To disseminate, cancer cells detach from the primary tumor, enter the blood stream and extravasate at distant organ sites such as the liver, lung, bone or brain. While cancer cells are known to evade contact inhibition during growth in culture, we found that cell density is still sensed and can signal through the Hippo pathway effectors LATS1 and YAP. These effectors control cancer cell invasive behavior into stromal tissues, expression of cytokines that recruit inflammatory cells and progression toward metastatic spread. In this perspective, we discuss the drivers and the significance of pathways controlled by cell growth density.

Cells sense their context in tissues and adapt to their microenvironment through autocrine and paracrine signaling. Upon malignant transformation, cells invade the local stromal tissue and enter the circulation by penetrating through the endothelial lining of the vasculature at the primary tumor site. These circulating cancer cells eventually extravasate and expand at distant organ sites where they form metastatic seeds. Metastatic spread initiates when the primary cancer is still below detection (<3 mm in diameter [1,2]) and is the major cause of disease recurrence, resistance to treatment, poor outcome and death from cancer. A number of signaling pathways have been identified including those that induce an epithelial–mesenchymal transition (EMT) signature associated with disease progression and metastasis and providing possible therapeutic targets. Here, we will focus on the contributions of Hippo pathway molecules to malignant progression. Recent studies indeed suggest that targeting the activity of the Hippo pathway transcriptional regulator YAP in combination with existing therapies could increase their efficiency and evade resistance [3]. For example, YAP acts as a survival input that counteracts the effects of MEK and BRAF inhibitors; thus, targeting YAP in combination with MAPK pathway inhibitors is an attractive therapeutic alternative strategy [4] and we will discuss their interconnectivity.

KEYWORDS

• cell density • cytokine signaling • Hippo pathway • vascular invasion

Cancer cells & contact inhibition

- In contrast to cancer cells, the growth of normal cells is limited through cell–cell contact inhibition.
- Still, cancer cells maintain some of their response to cell density.

Once normal epithelial cells have reached confluence, they stop proliferating. On the other hand, cancer cells continue growing, are refractory to contact inhibition and typically display anchorage-independent growth in suspension. Interestingly, hypersensitivity to contact inhibition of cells from the naked mole rat is attributed to the longevity of this species and its striking

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resistance to cancer [5]. Although loss of contact inhibition is one of the distinctive features of malignant transformation [6], cancer cells still sense contact with neighboring cells and respond in various ways depending on the context and the signaling pathways involved. We have found that the density at which cells are grown in tissue culture regulates the vascular invasiveness of frequently studied cancer cell lines. Cells grown at high density were less successful at invading an endothelial monolayer *in vitro* and at metastasis *in vivo* than cells grown at low density [7]. Quite strikingly, several known aggressive cancer cell lines showed highly malleable invasive behavior in response to altered cell density in culture (summarized in Table 1).

The Hippo pathway & cancer

- The Hippo pathway plays a crucial role during development and tissue homeostasis. Deregulation of this pathway has been implicated in cancer initiation and progression.
- Cell compaction during development acts as a regulating signal and uses the Hippo pathway.
- The Hippo pathway controls cancer cell metastatic spread in response to different cell density in culture.
- *KRAS* oncogenic signaling utilizes *YAP* during malignant progression.

The Hippo pathway and its interconnections (Figure 1) were unraveled during the past years as one of the major signaling pathways that controls organ size and is altered by cell density. Studies in flies and mammals established a conserved role for the *Drosophila* Hippo kinase

and the mammalian equivalent, STE20-like protein kinases (Mst1/2) as regulators of organ size. Moreover, the pathway has been shown to function as a tumor suppressor in several cancers [8,9]. Although the core Hippo pathway components are conserved, their functions vary in different tissues, contexts and model systems [10]. The LATS kinase phosphorylates and negatively regulates the activity of the transcriptional modulators YAP and tafazzin (TAZ) that are considered as the major transducers of Hippo pathway activity [11]. Although their role as transcriptional co-activators is well established, recently Kim *et al.* have also shown a co-repressor function for YAP and TAZ that promotes cell growth and survival [12]. An ever increasing number of upstream extracellular signals such as cell density, mechanical sensing and G-protein-coupled receptors, to name but a few have been shown to be integrated via YAP/TAZ transcriptional regulation during organ growth and tissue homeostasis (Figure 1) [13–15]. Deregulation of YAP signaling is associated with several diseases including cancer [16–18] and YAP has been considered as an appealing therapeutic target since it is found upregulated in different types of cancers. Also, YAP expression or activation has been shown to correlate with poor disease outcome [11,19–20]. YAP transcriptional activity is also associated with metastasis of melanoma, lung and breast cancer [21–23]. In pancreatic cancer that is driven almost exclusively by mutant *KRAS*, the laboratory of Chunling Yi at Georgetown University showed that YAP is rate limiting for malignant progression irrespective of the presence of mutant P53 [24]. Complementary to this, YAP expression appears to be sufficient to overcome the loss of *KRAS*

Table 1. A list of cell lines and their invasive behavior upon growth at different cell density.

Cell line	Cancer type	Invasion (%) at cell density	
		High	Low
MDA-MB-231	Human breast	7.42	36.41
MDA231-L	Human breast	8.59	26.94
MDA231-Br	Human breast	5.14	28.48
MDA231-Bo	Human breast	4.65	18.79
U87-MG	Human brain	25.83	42.21
PC3	Human prostate	17.94	30.88
THP1	Human leukemia	15.26	30.12
COLO357	Human pancreas	44.52	42.83
E0771	Murine mammary	28.14	41.12

MDA231-L, -Bo, -Br are MDA-MB-231 cells that have been selected to metastasize to the lung, bone and brain, respectively, and were provided by the Massague laboratory.
Data taken from [7].

Figure 1. Schematic of the Hippo pathway and some interactions in mammalian cells. Signals from the cell membrane either activate or inhibit the Hippo pathway depending on the cellular of tissue context and upstream regulators. YAP and TAZ (transcriptional co-activator with a PDZ-binding domain) co-activate TEAD (TEA domain family members) transcription factor target genes. The Hippo core (gray shade) molecules function as regulators of cellular location, abundance and activity of YAP/TAZ through different mechanisms including direct phosphorylation by the Ser/Thr LATS kinases (large tumor suppressor; *Drosophila* Warts). Phosphorylation of YAP/TAZ controls cytoplasmic retention via 14-3-3 interaction or degradation, thus reducing transcriptional activity of YAP/TAZ. MST1/2 (STE20-like protein kinase 1/2) are the homologs of *Drosophila* Hippo that provided the name for the pathway. SAV1 (Salvador family) are WW domain-containing proteins, MOB1/2 is a kinase activator. In addition to the above regulation, YAP can be sequestered by CRBs (crumbs family members) in a complex with AMOT (angiomin). GPCR: G-protein-coupled receptor.

signaling in KRAS-dependent cancers [25]. In addition, Hong *et al.* have shown a positive feedback loop where KRAS stabilizes YAP to ensure progression toward transformation [26,27].

The Hippo pathway has been linked to several aspect of metastasis. Lamar *et al.* have shown that YAP interaction with the TEAD/TEF family of transcription factors strongly enhances metastasis in breast cancer and melanoma cells [21–23]. Another study suggests a proinvasive role for

both Hippo pathway effectors YAP and TAZ in cutaneous melanoma [28]. In lung cancer, knockdown of YAP and TAZ reduced cell migration and transplantation of metastatic disease *in vivo* [21,23]. Also, a missense mutation in the *YAP1* gene appears to increase the risk for lung adenocarcinoma [29]. Our group has shown the contribution of the Hippo pathway to cancer cell/endothelial crosstalk and vascular invasion. Quite surprisingly, some of the most aggressive cancer

cell lines can switch to a noninvasive phenotype when sensing a high growth density in culture. This phenomenon is reversible when growth conditions are switched back to low density. We observed that cancer cells grown at low density present a highly invasiveness phenotype through upregulation of several cytokines including *IL8*, *CXCL1*, 2 and 3 as YAP appear to translocate to the nucleus. Inhibition of YAP–TEAD association using the inhibitor Verteporfin reversed this upregulation [7]. These findings matched with the YAP-dependent control of inflammatory cytokine expression and signaling in the mouse model of pancreatic cancer driven by mutant *KRAS* mentioned above [24].

Beyond its role in cancer, Hippo pathway signaling is engaged early on during embryo development. Activity of the pathway impacts cell fate decisions in the inner cell mass relative to the surface trophoectoderm that forms the placenta in mammals. During embryonic inner mass development, YAP cellular localization pattern impacts signaling molecules that control cell polarity and cell–cell crosstalk (Figure 2). The less compacted cells with nuclear YAP localization, give rise to the trophoectoderm while the more compacted cells in the inner cell mass show cytoplasmic localization of YAP [15,30–31].

This is somewhat reminiscent of observations during malignant progression [7]: YAP cellular localization regulates the invasive capability of cancer cells and cancer cells forming lung metastases at the endothelial boundary of blood vessels showed nuclear YAP staining in contrast to cells distant from the endothelial invasion zone (Figure 2). Quite strikingly, signaling molecules involved in organ size control mechanisms coordinated by YAP also appear to contribute to invasive cancers [32–34].

Cytokines & invasion

- Increased cytokine levels are associated with aggressive cancer phenotype, metastasis and poor prognosis.

High levels of cytokine expression have been associated with disease progression and cytokines levels were found elevated in metastases when compared with biopsies of primary tumors. Moreover, low levels of cytokines were associated with a longer relapse-free patient survival supporting their impact on the aggressiveness of the particular cancer [35]. In mechanistic studies with cultured cells and animal models, cytokines were associated with a more invasive cancer cell phenotype. Also, cancer cells that express *CXCL1*

Figure 2. YAP cellular localization determines cell behavior in early development and metastasis. (A) Scheme of a blastocyst stage showing nuclear YAP (red) in the less compacted cells forming the outside invasive trophoectoderm layer versus cytoplasmic YAP in the more compacted cells in the inner cell mass (modified from [30]). (B) Schematic and (C) stain of lung metastases for YAP protein. Nuclear YAP staining is seen in cancer cells in small, recently established metastases close to blood vessels. Cytoplasmic YAP staining is seen in the larger established metastasis. A quantitation showing a significant difference and the original data are in [7].

Figure 3. Model that connects cancer cell growth density and regulation of vascular invasion via LATS1-YAP signaling through the control of cytokine expression and signaling.

Adapted from [7,24].

and 2 attract mesenchymal cells in response to the cytokines and render the cancers more metastatic and resilient to chemotherapy [36]. In addition, IL-6 has been shown to activate an inflammatory response in breast cancer that result in chemotherapy resistance and expansion of the cancer stem cell population [37]. In support of their role, several cytokines including the above CXCL1 and 2 and IL-6 plus IL-8 were found upregulated in lymph node metastases from a triple negative breast cancer cell line xenograft [38]. Furthermore, circulating cancer cells are attracted into developing metastases by their cytokine expression gradient, thus expanding the lesion through self-seeding of cancer cells [39]. Additionally, activation of CXCR2/4 pathways due to TGF- β signaling loss attracts mesenchymal cells that promote

metastasis [40]. This recruitment of inflammatory cells offers more cytokine activity that augments cancer cell survival and an increase of the stem cell population [40,41] ultimately leading to poor prognosis and to treatment resistance [36]. We have shown that blocking CXCR2 signaling, the receptor for many of the cytokines upregulated in cancer cells grown at low density, inhibited the migration and endothelial monolayer invasion *in vitro* as well as extravasation of invasive cancer cells *in vivo* [7]. Also, inhibition of YAP activity inhibited the expression of those cytokines. Thus, vascular invasion of cancer cells grown at different densities is mediated to a significant extent through CXCR2 autocrine and paracrine acting cytokines whose expression is regulated by YAP (Figure 3) [7].

Our group and others have shown that increased expression of IL-6 [37] or IL-8 [42] is associated with EMT signatures. In fact, these cytokines enhance EMT which was also observed under low cell density growth conditions. We have reported that E-cadherin, an epithelial marker, is upregulated in MDA-MB-231 cells when grown at high density, noninvasive conditions yet E-cadherin levels are not detectable when those cells are cultured at low cell density corresponding with a mesenchymal signature. The E-cadherin upregulation coincides with YAP phosphorylation, cytosolic retention of YAP and a loss of the invasive phenotype. Complementary to this observation, Kim *et al.* [43] showed that exogenous overexpression of E-cadherin in MDA-MB-231 cells resulted in the nuclear exclusion of YAP.

The Hippo pathway signaling was shown also to contribute to EMT through the TGF- β -SMAD pathway [44]. Secreted factors affecting the cancer cell/stromal crosstalk can modulate EMT creating a cancer cell stem niche [41]. Moreover, Oskarsson *et al.* [45] have shown that Tenascin C, an extracellular matrix protein, facilitates metastatic seeding of lungs by breast cancer cells using stem cell signaling paths that include *musashi* homolog 1. We found Tenascin C upregulated in the invasive MDA-MB-231 cell population which were cultured at low growth density.

In addition to a regulation of proteins, we found that in invasive cells upon YAP transcriptional activation the miR-29b pathway was downregulated [46]. Interestingly, miR29b is known to be a suppressor of angiogenesis and metastasis. Thus, its downregulation would match with its contribution cancer cell vascular invasion.

Conclusion

Cancer cells are refractory to contact inhibition and the mechanism by which normal cells halt divisions at high confluence is no longer

functional in cancer cells. Still, altered cell–cell contacts between cancer cells growing at different density provide cues to the cells that controls their crosstalk with the stroma and ultimately vascular invasion and metastatic spread. Thus, the invasive and metastatic phenotype of cancer cells can be regulated independently of their growth phenotype is susceptible to cell growth density and modulated through elements of the Hippo pathway [7].

Future perspective

The Hippo pathway acts as a sensor to external stimuli such as growth density of cultured cells and compaction *in vivo* by controlling gene expression including secreted cytokines. Those factors contribute to the metastatic progression and can be targeted by blocking antibodies or pathway inhibitory drugs. Furthermore, the crosstalk between established oncogenic drivers such as mutant *KRAS* and the Hippo pathway suggest a potential for combined therapy. More recently, TAZ a Hippo pathway transducer was targeted with statins in a clinical trial for breast cancer patients (NCT02416427). CXCR2 is also an attractive therapeutic target that has been entered into several clinical trials in melanoma and pancreatic cancers (NCT01740557, NCT00851955).

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EXECUTIVE SUMMARY

- Cell–cell contact inhibition limits the growth of normal cells.
- The Hippo/YAP pathway integrates cell–cell crosstalk and controls growth density in cell culture as well as organ size.
- Cell–cell contact growth inhibition is lost in cancer cells.
- The Hippo/YAP pathway in cancer cells senses cell growth density and regulates the invasive phenotype of cancer cells.
- Cytokine expression controlled by cell density and Hippo pathway signaling is rate limiting for cancer cell vascular invasion and metastasis.

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