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Emerging targets: Molecular mechanisms of cell contact-mediated growth control

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Emerging targets: Molecular mechanisms of cell contact-mediated growth control. Contact inhibition of cell proliferation evokes a unique cellular program of growth arrest compared with stress, age, or other physical constraints. The last decade of research on genes activated by cell-cell contact has uncovered features of transmembrane signaling, cytoskeletal reorganization, and transcriptional control that initiate and maintain a quiescent phenotype. This review will focus on mechanisms controlling contact inhibition of cell proliferation, highlighting specific gene expression responses that are activated by cell-cell contact. Although a temporal framework for imposition of these mechanisms has not yet been well described, contact inhibition of cell proliferation clearly requires their coordinated function. Novel targets for intervention in proliferative disorders are emerging from these studies.

The capacity for cells to judiciously regulate proliferative responses in the context of cell-cell contact is a fundamental requirement for the organization and maintenance of specialized tissues in multicellular organisms. Indeed, contact-mediated inhibition of growth is not restricted to cells in tissues but is a general property displayed by cultured primary cells of many types, including fibroblasts, smooth muscle, endothelial, epithelial, and other lineages. Increasing cell-cell contact in adherent cells eventually blocks the growth factor and integrin-mediated stimuli to proliferate in vitro. This contact or density-dependent inhibition of cell proliferation (herein referred to as contact inhibition) is achieved at a cell-culture specific saturation density.

One parameter that distinguishes cancer-derived transformed cells from primary cells cultured from normal tissue is the loss of contact inhibition [1, 2]. In fact, an important window into understanding contact inhibition has been the identification of specific mRNA transcripts that are induced by cell-cell contact in normal, but not transformed, cells. Reports on genes that are activated at contact inhibition (summarized in Table 1) suggest that the cellular program of growth arrest controlled by cell-cell contact is unique compared with the growth arrest induced by stress or age. Expression of growth arrest-specific (GAS) or growth arrest- and DNA damage-inducible (GADD) genes is induced by both serum starvation and contact inhibition [3], or by both serum starvation and DNA-damaging agents, respectively [4]. Although the growing list of genes activated by contact inhibition include the GAS family by definition, a number of important discoveries include genes that are not activated by stress. The degree to which the cellular program of contact inhibition will diverge from GAS genes, GADD genes, growth arrest imposed by limiting cell-extracellular matrix surface area contact [5] or cell senescence [6] remains to be determined. This review will discuss the molecular mechanisms controlling the initiation and maintenance of contact inhibition (Fig. 1).

WHICH CELL-CELL CONTACT IS MOST IMPORTANT?

Theoretically, contact inhibition could be controlled by a limited number of, or possibly one, cell surface molecule(s). However, recent reports reveal that loss of specific cell surface moieties or disruption of any one cell-cell contact structure may be singularly capable of releasing cells from contact inhibition. For some time, it has been known that contact inhibition-induced gene expression and synthesis of plasma membrane gangliosides is lost in transformed cells [7]. Exogenous addition of soluble gangliosides suppress proliferation by inhibiting RPTK (receptor protein tyrosine kinase) activation [8–11]. A possible mechanism for this RPTK inhibition was lacking until it was shown that return of contact inhibition in A431 cells by ganglioside G_{M3} is mediated through RPTP-σ (receptor protein tyrosine phosphatase) [12]. Thus, heterotopic interaction between upregulated gangliosides and upregulated RPTPs (see Table 1) on adjacent cells may be a key mechanism of growth arrest.

Key words: contact inhibition, growth arrest, quiescence, review.

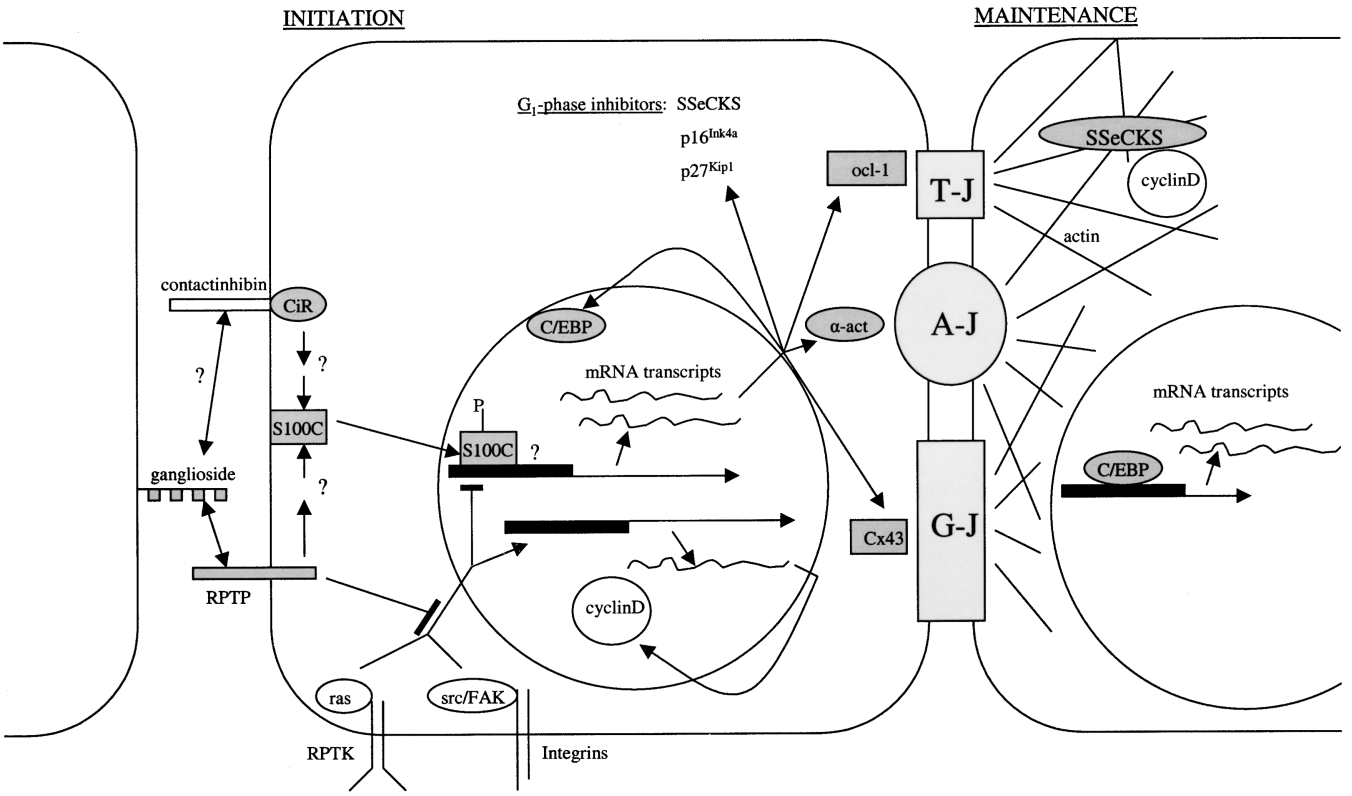

Fig. 1. A proposed model integrating specific molecular mechanisms that initiate and maintain contact inhibition. INITIATION: Activation of receptor protein tyrosine phosphatases (RPTP) and contactinhibin by cell surface molecules on adjacent cells inhibits mitogenic pathways downstream of receptor protein tyrosine kinase- (RPTK) and integrin-activated kinases such as *ras* and *src*. This leads to the suppression of cyclin D expression and transcriptional activation of *ras*- and *src*-suppressed genes encoding cell-cell contact proteins, including occludin-1 (ocl-1) in tight-junctions (T-J), α-actinin (α-act) in adherens-junctions (A-J), and connexin43 (Cx43) in gap-junctions (G-J), favoring cytoskeletal polymerization between affinity partners. G_1-phase inhibitors are also up-regulated and may additionally be transactivated by phosphorylated (*P*) S100C. MAINTENANCE: C/EBP transcription factors contribute to ongoing mechanisms from the initiation phase by activating genes that lead to a fully differentiated and quiescent phenotype.

Contactinhibin was similarly discovered to control contact inhibition through assays that challenge proliferating fibroblasts with cell membrane fractions [13]. Unlike membrane gangliosides, however, the soluble form of contactinhibin, a 60–70 kD membrane glycoprotein, does not inhibit growth, nor is its cell surface expression lost with transformation. The inhibitory activity of contactinhibin is delegated to its "downstream" membrane receptor, CiR, which is downregulated and hyperphosphorylated in transformed cells [14].

In fact, aberrant expression or function of a number of "downstream" cell-cell structural proteins can singularly release cells from contact inhibition. Reviewed elsewhere [15, 16], the importance of adherens-junctions in growth control is well-described. The function of adherens-junctions in contact inhibition is to regulate the activity of β-catenin, as modest overexpression of β-catenin is sufficient to maintain proliferation at high cell density [17]. However, less attention has been focused on the role of gap-junctions and tight-junctions. Oligonucleotide antisense-inhibited expression of connexin43, a *ras*-suppressed gap-junction protein [18], resulted in significantly higher saturation densities in BALB/c 3T3 cells [19]. Re-expression of connexin43 reverted transformed cells to contact inhibited growth marked by dye coupling between cells [20]. Using similar methodology, a recent study showed that the re-expression of occludin-1, a *raf*-suppressed tight-junction protein, resulted in return of contact inhibition in transformed salivary epithelial cells [21]. This coincided with recovery of claudin-1 protein levels, relocation of ZO-1 to tight-junctions, and redistribution of E-cadherin to lateral membranes. Moreover, the expression and assembly of tight-junction proteins following disruption of *raf* pathways using MEK1 inhibitors in *ras*-transformed epithelial cells was found to precede the appearance of E-cadherin-based junctions [22]. This challenges the belief that adherens-junction is the primary cell-cell contact structure mediating contact inhibition. In total, these observations strengthen the notion that contact inhibition requires the coordinated function of a number of cell-cell contact proteins.

Table 1. Genes up-regulated at contact inhibition

Gene	Proposed mechanism of action	Reference
Secreted factors		
Acidic-FGF	Survival/anti-apoptotic factor	[62]
Clusterin	Heterodimeric glycoprotein promoting anti-apoptosis	[63]
Decorin	Binds TGF-β and thrombospondin, and is a novel ligand for EGF receptor, leading to $p21^{Waf1}$ upregulation	[64, 65]
GAS6	Vitamin K-dependent ligand (related to protein S) of *Axl* family of receptor protein tyrosine kinases, inhibiting apoptosis	[66–68]
p20K	Member of the lipocalin family of lipid-binding proteins, resulting in enhanced transport of polyunsaturated fatty acids	[56]
Non-secreted factors		
Cell cycle components		
Cyclin D2	Sequestration of the CDK2 catalytic subunit	[69]
$p16^{Ink4a}$	Inhibition of CDK4 and 6, with $p16^{Ink4a}$ −/− cells exhibiting higher saturation density but no foci formation compared with $p16^{Ink4a}$ +/+ cells	[40, 41]
$p27^{Kip1}$	Inhibition of CDK2, 4, and 6, however $p27^{Kip1}$ −/− cells exhibit no higher saturation density or foci formation compared with $p27^{Kip1}$ +/+ cells	[39, 42]
Transcription or translation factors		
C/EBP-β	Leucine-zipper type transcription factor that activates a "quiescence-responsive unit"	[56]
C/EBP-δ	Leucine-zipper type transcription factor that activates genes associated with growth arrest and differentiation	[55]
C/EBP-ζ	Leucine-zipper type transcription factor that activates genes associated with growth arrest and differentiation	[55]
GAS5	Non-protein-coding multiple small nucleolar RNA (snoRNA) involved in growth-dependent regulation of protein synthesis	[70]
Transmembrane phosphatases or kinases		
GAS9	PDGFα-receptor that promotes cell "competence" for cell cycle progression	[71]
HPTP-β	Enhanced PTPase activity	[72]
PTPRJ	Enhanced PTPase activity	[51, 52]
RPTP-σ	Enhanced PTPase activity	[73]
RPTP-μ	Enhanced PTPase activity	[74]
Cytoskeletal proteins		
Caveolin	Signal molecule scaffolding protein in caveolae that negatively regulates the ERK1/ERK2 MAP kinase cascade	[30, 31]
GAS1	Novel integral membrane protein of unknown function; however, antisense RNA-inhibited expression induces high saturation density	[75]
GAS2	Actin-associated protein involved in microfilament reorganization	[76]
GAS3	Transmembrane glycoprotein of unknown function associated with hereditary proliferative neuropathies	[77]
MARCKS	F-actin cross-linking in cytoskeletal reorganization	[78, 79]
p18H-rev107	*ras*-suppressed gene of unclear function	[80]
SSeCKS	Cytoplasmic sequestration of G_1 phase cyclins and scaffolding of signaling molecules	[32, 36]
Other signaling proteins		
Neurofibromin	Increased GTPase activating protein activity, maintaining differentiation	[81]
PC5	Subtilisin/kinin-like endoprotease that cleaves trans-membrane phosphatases	[82]
PI 3-kinase	Role unclear	[83]
PKC-α	Role unclear	[78]
PKC-δ	Role unclear	[78]
S100B	EF-hand Ca^{2+}-binding protein that facilitates nuclear accumulation of p53	[59]
S100C	EF-hand Ca^{2+}-binding protein with phosphorylation dependent cytoplasmic-to-nuclear translocation, leading to $p16^{Ink4a}$ and $p21^{Waf1}$ up regulation	[57]

"CLASS II" TUMOR SUPPRESSORS: CYTOSKELETAL CONTROL OF CONTACT INHIBITION

Studies on "class II" tumor suppressors, genes that are not altered at the DNA level ("class I") [23], have further highlighted the importance of the cytoskeleton in contact inhibition. Similar to occludin-1, a number of cytoskeletal proteins (examples: α-actinin, tropomysin, gelsolin [24–26]) are transcriptionally downregulated in transformed cells and establish contact inhibition when reexpressed. The paradoxical dominance of one reexpressed cytoskeletal protein over signaling pathways may be by favoring cytoskeletal polymerization between affinity partners, as has been modeled for focal adhesion assembly [27]. This new cytoskeletal network likely impacts aberrant signaling by normalizing spatial-temporal constraints on signaling proteins that are organized by the cytoskeleton [28]. Direct evidence for a similar mechanism in contact inhibition has come with recent reports on two cytoskeletal scaffolding proteins.

Caveolin, the principal membrane protein component of caveolae, has been characterized as a "general kinase inhibitor" based on the observation that its signal molecule scaffolding domain inhibits a number of signal transduction pathways [29]. Interaction between the caveolin cytosolic membrane-proximal scaffolding domain and the caveolin-binding motif in a number of signaling molecules, including G-protein α subunits, H-Ras, Src family tyrosine kinases, PKC isoforms, EGF-R, Neu, and eNOS, inhibits their activity. Similar to other "class II" tumor-suppressing cytoskeletal proteins, caveolin-1 mRNA and protein are downregulated in transformed cells, and re-expression establishes contact-inhibited growth. Recently, it was discovered that caveolin-1 expression is upregulated by cell-cell contact, whereas antisense RNA-inhibited expression in NIH3T3 cells was sufficient to constitutively activate the ERK1/ERK2 MAP kinase cascade with loss of contact inhibition [30]. This upregulated expression was marked by subcellular redistribution of caveolin-1 to sites of cell-cell contact [31].

SSeCKS similarly controls mitogenic pathways by scaffolding signaling molecules in the cytoplasm. However, unlike caveolin-1, the upregulated expression of SSeCKS at contact inhibition leads to its direct interaction with cell cycle components [32]. Detected in screens for *src*- and *ras*-suppressed "type II" tumor suppressors [18, 33], SSeCKS is a major PKC substrate with multivalent scaffolding activity for PKC, PKA, and Ca^{2+}-calmodulin [34, 35]. Inducible ectopic expression in subconfluent cells disengaged SSeCKS from its cell-cycle-regulated expression [36] resulting in growth arrest in G_1 [32]. This was accompanied by elaboration of SSeCKS-staining cell processes [37], mimicking the expression of SSeCKS in morphologically differentiating cells during embryogenesis [38]. Importantly, the G_1 arrest in subconfluent SSeCKS overexpressors recapitulated the cell cycle control by SSeCKS at contact inhibition by at least two mechanisms: ERK2-dependent decreases in cyclin D, and cytoplasmic sequestration of G_1-phase cyclins by interaction with SSeCKS' cyclin binding motifs [32]. SSeCKS' up-regulation at contact inhibition is similar to two other G_1-phase inhibitors, $p27^{Kip1}$ and $p16^{Ink4a}$ [39, 40]. However, only loss of $p16^{Ink4a}$ or SSeCKS function [32, 41], not $p27^{Kip1}$ [42], results in higher saturation densities, suggesting that some G_1-phase inhibitors are more critical for contact inhibition. Accumulation of hypophosphorylated Rb in growth-arrested subconfluent SSeCKS overexpressors [32] or confluent fibroblasts [43] is consistent with the need for active transcriptional repression by complexed Rb-E2F to initiate contact inhibition [44]. This suggests that the contact inhibition observed in cells deleted for specific Rb isoforms is through compensation from other Rb family members [45]. Thus, caveolin and SSeCKS act at the intersection of cytoskeletal reorganization and mitogenic control in contact inhibition.

STUDIES IN ENDOTHELIUM: CELL SURFACE SIGNALING IN CONTACT INHIBITION

During angiogenesis, inhibitory signals that maintain the contact inhibited network of endothelium in a parent vessel must be overcome to contribute cells to new outgrowth. This model is supported by observations that both tyrosine phosphatase activity and RPTPs are up-regulated, and intracellular mitogenic pathways inhibited, in confluent endothelial cells. Endothelial cells, along with other cell types, exhibit higher membrane phosphatase activity at confluence than during subconfluent growth or growth arrest by serum starvation [46–49]. Mitogenic pathways are inhibited downstream of Ras-MEK1 in confluent endothelial cells [50]. Moreover, addition of the phosphatase inhibitor, sodium orthovanadate, to confluent endothelial cells activates the ERK1/ERK2 MAP kinase and PI 3-kinase/Akt pathways, followed by cyclin D expression and $p27^{Kip1}$ downregulation [49]. Therefore, upregulated RPTPs likely attenuate key mitogenic pathways at contact inhibition. However, little is known about how these RPTPs are activated by cell-cell contact.

Studies on PTPRJ (originally called density enhanced phosphatase-1 [51]), a 220 kD RPTP abundantly expressed in the endothelium of the kidney, provide evidence for how RPTPs may be activated at contact inhibition [52]. In primary human renal microvascular endothelial cells, PTPRJ clusters at sites of cell-cell contact, suggesting that cell-contact-induced oligomerization may be important for activity. This notion was supported by observations that oligomerizing bivalent antibody directed against PTPRJ ectodomains arrested subconfluent endothelial cell proliferation in the presence of growth factors, whereas monovalent antibody had no effect. Cells were blocked from entering the G_1/S transition without increased apoptosis. Importantly, FGF-induced angiogenesis, in an in vivo mouse corneal pocket assay, was blocked by bivalent antibody, but not by monovalent antibody. Thus, loss of contact inhibition in the parent vessel likely required disruption of oligomerized PTPRJ. Interestingly, challenging proliferating endothelial cells with endothelial membrane fractions arrest growth [53]. A key study will be to discover the factor on adjacent endothelial cell membranes that causes PTPRJ oligomerization.

WHAT INITIATES GENE EXPRESSION AT CONTACT INHIBITION?

Immediate phosphatase inactivation of mitogenic signaling at contact inhibition likely leads to expression of some genes. However, additional transcriptional mechanisms are emerging. C/EBP (CCAAT/enhancer-binding protein) transcription factors are important in the differentiation and function of many tissues [54]. Two reports have shown that certain C/EBP isoforms are upregulated at contact inhibition in specific cells: C/EBP-δ and C/EBP-ζ

in mammary epithelial cells and NIH3T3 cells, respectively [55], and C/EBP-β in chicken embryo fibroblasts [56]. Although transcriptional targets were not identified for C/EBP-δ and C/EBP-ζ, antisense RNA inhibition of C/EBP-δ expression delayed growth arrest [55]. However, a C/EBP-β targeted "quiescence-responsive unit" was mapped in the GAS gene, p20K, raising the possibility that similar sequences may exist in the promoters of other genes activated by contact inhibition [56]. Yet, an enigma remains: Given that C/EBP isoforms are themselves transcriptionally activated by contact inhibition, what controls C/EBP expression? A possible temporal mechanism is exemplified by the activity of S100C during contact inhibition [57]. Selected for study based on its downregulated expression in immortal fibroblasts, S100C, an EF-hand Ca^{2+}-binding protein similar to calmodulin [58], was found to undergo phosphorylation on threonine 10 followed by cytoplasmic-to-nuclear translocation in normal, but not immortal, cells as they became confluent. Injection of anti-S100C antibody into confluent cells initiated DNA synthesis, whereas forced nuclear accumulation of chimeric S100C-nuclear localizing protein inhibited DNA synthesis marked by upregulation of $p16^{Ink4a}$ and $p21^{Waf1}$. S100C may shuttle growth-regulatory proteins between the cytoplasm and the nucleus, similar to how S100B [59] and calmodulin [60] facilitate p53 and cyclin D translocation, respectively. However, based on preliminary DNA binding observations, the authors speculate that S100C has transcriptional activity like another EF-hand Ca^{2+}-binding protein, DREAM [61].

FUTURE DIRECTIONS

The finding that both S100C and S100B are also upregulated by contact inhibition [57, 59] point to the fact that the cell-cell contact-induced intermediaries regulating growth arrest remain poorly understood. Does upregulation of these EF-hand Ca^{2+}-binding proteins occur via auto-regulatory loops, or is another upstream factor involved? There is likely to be significant cross talk and hierarchical control between mechanisms in contact inhibition. Future research to discover how these mechanisms interact with each other will provide additional targets for intervention in proliferative disorders.

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