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TRENDS

Gradient sensing in vectorial chemotaxis – a novel role for Reactive Oxygen Species

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ABSTRACT

Reactive oxygen and nitrogen species (ROS and RNS), once condemned as universal nemeses, are widely accepted today as powerful allies to the functional repertoire of a cell. ROS as signaling molecules mediates cellular proliferation, senescence, apoptosis, immune response, cytoskeleton remodeling and migration. Here, we examine the role of these in directional chemotaxis, a fundamental yet relatively novel platform for ROS based innate immune mechanisms.

Keywords: Reactive oxygen species (ROS), chemotaxis, GTPase, actin, extra-cellular superoxide dismutase (ECSOD)

INTRODUCTION

Chemotaxis is defined as the migration of cells along a concentration gradient. This cellular phenomenon is commonly seen in endothelia, neutrophils and monocyte-macrophages. Effects in differentiated tissue are subtle, as opposed to dramatic migrations in response to morphogen gradients that symbolize embryonic tissue. Innate immunity is a set molecular and cellular events that are initiated by a breach in integrity of anatomical and physiological barriers that form the first line of host defense. Inflammatory stimuli (infection or injury) result in the progressive movement of neutrophils towards the offending trigger. This follows an increase in vascular permeability with appearance of inter-endothelial gaps, diapediasis, extravasation and transmigration (Figure 1). This is accomplished by extending lamellipodia at the leading edge with concomitant retraction posteriorly. The formation of membrane bound cytoplasmic extensions is however, inherently stochastic, with several lamellipodia developing in parallel. Cycles of positive feedback ensure fine tuning this response to a single dominant extension with consequent vectorial movement. Critical to this response is the ability to sense chemically defined gradients. The importance of this subtle variation in concentrations may be gauged by morphogen induced patterning of developing tissue.

Organized cells frequently exhibit binary behavior, switching between a subthreshold 'none' and a suprathreshold 'all' states.. In contrast, the event space in a quantum environment is infinite and inherently more complex to comprehend. Several workers are racing to resolve signal transduction mechanisms that govern these response patterns. The ubiquitous production of reactive oxygen species, in tandem with metabolic intermediates offers an elegant solution to this problem. Fundamental to this process is the dynamic fusion and fission of numerous scaffolds, domains and clusters of proteins and membranes. In this treatise, we review literature relevant to the development of the cell as a stable sensing tool.

FOCAL ADHESIONS AND INTEGRINS

Focal adhesions (FAs) are transient units of cell-matrix connectivity.[1] These begin as complexes and progress to stable adhesions (focal and fibrillar).[2–4] A representative unit comprises clusters of integrin heterodimers (permutations of 18 α and 8 β subunits that facilitate inter-cellular, cell-matrix and cell-pathogen interactions),[5] integrin binding proteins such as talin,[6] adapter proteins (paxilin,[7] vinculin,[8] and α-actinin)[9] and enzymes. These include tyrosine kinases (e.g., focal adhesion kinase (FAK)[10] and proline-rich tyrosine kinase-2 (Pyk2)),[11] serine/ threonine kinases (e.g., integrin linked kinase (ILK),[12,13] p21-activated kinase (PAK)[14] and phosphatidyl-inositol-3 kinase (PI3K),[15] phosphatases (PTEN))[16] and protein tyrosine phosphatase (PEST).[17] In addition to single proteins, pre-assembled complexes are also recruited to focal adhesions (PINCH- ILK-α parvin ternary complex).[18,19]

Ruffles, are membrane patches that overly regions of heightened cytoskeletal remodeling. Rac[20] and Rho[21] are small GTPases in tandem with focal complexes/ adhesions that regulate this cellular restructuring in response to stimuli. The asymmetric distribution and downstream effectors of Rac1 and RhoA ensure the generation of a cellular bias.[22] Thus, while Rac1 promotes leading edge activity by local actin polymerization events,[23,24] RhoA is found in Rac1 deficient zones and causes actin disassembly with subsequent posterior edge retraction. Initial focal complex dynamics are mediated by Rac1 and Cdc42, while stabilization is brought about by RhoA.[2,3] The mechanisms of Rac1 induced actin reorganization involve a complex interplay of factors and includes activation-internalization of membrane bound receptors and microdomains,[25] production of ROS and RNS,[26] and several downstream protein-protein interactions.

ROS AND CELLULAR MICRO-ORGANIZATION

Reactive oxygen species, a molecular ensemble of free radicals and metabolic intermediates comprises superoxide anions ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radicals (·OH) and ions and usually occurs in tandem with reactive nitrogen species (RNS). ROS are generated by the actions of NAD(P) H oxidase, superoxide dismutase, Fenton's chemistry, xanthine oxidase, myeloperoxidase and nitric oxide synthase (NOS). These reactive and short lived molecules are increasingly being viewed as critical regulators of signal transduction. The reader may wish to consult excellent reviews for details of ROS as signaling messengers.[27–30] ROS in non-phagocytes are intracellular, lower in concentration, the $p47^{phox}$ analog (NOXO1) is constitutively active and substrates include NADH or NADPH. In addition, there is a slow sustained rise, a delayed peak and potential for repeat cycles of superoxide production unlike that seen in neutrophils. Superoxide anions are reactive and short lived.

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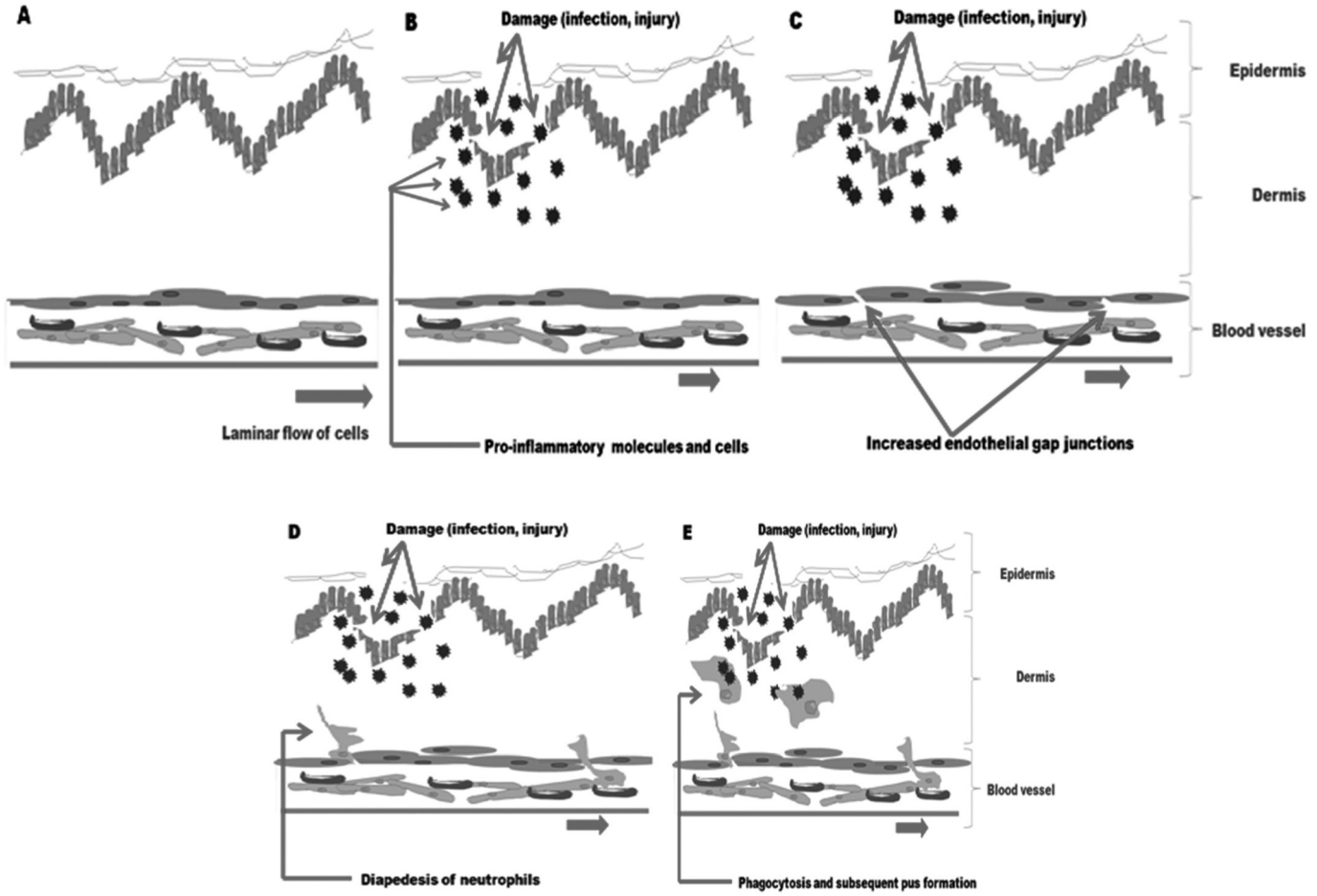

Figure 1 | Temporal evolution of inflammation in response to stimuli A) cross-section of skin and subcutaneous tissue, B) damage to tissue with release of noxious stimuli, C) Increased permeability of vasculature due to widening of inter-endothelial gaps, D) withdrawal of neutrophils from laminar vascular flow with increased adhesion and diapedesis, E) extravasation and migration of phagocytic competent cells towards chemo-attractants.

These dismutate spontaneously or enzymatically to hydrogen peroxide, are complexed to nitric oxide or transported intracellularly by the chloride anion transporter (ClC3).[31,32] A similar cycle of generation, transport, propagation and termination has been proposed for intracellular ROS and endosomal signaling.[33] In conjunction with Rac, Rho and Cdc42 proteins, several investigators have identified redox sensitive periodic restructuring of the actin[34–40] and microtubular[41,42] cytoskeletal elements as an important initial event in a sequence terminating in cell motility, extravasation, metastasis, trans-differentiation, interactions with extra-cellular matrix and inter-cellular communication.

Mechanistic details of actin-centric movement involve the integration of numerous dynamic signaling platforms that function to transduce extra-cellular signals into appropriate migratory cues. These effector multi-protein complexes include cofilin,[43] gelsolin,[44–47] filamin,[48–50] and the non-receptor tyrosine kinases (FAK,[51,52] Pyk2[53,54]) and function to stabilize or dismantle networks of actin. As discussed previously, stimuli often include physical forces (e.g. light, mechanical stretch), growth factors, small signalling molecules such as ROS and RNS.[55–57] In neutrophils, chemotatic movement is preceded by a sequence of polarizing events chiefly orchestrated by alternate cycles of Rac and Rho activity.[58–60] This results in a transient state of the cell characterized by the presence of multiple lamelipodia (Figure 2). The exact function of these cytoplasmic extensions is not known but, they may serve as chemical antennae. The cell is thus, able to sense and eventually seek optimal concentrations of stimulating molecules. Early work showed that chemo-attractants could interact with GPCRs[22] and recruit phospatidylinositide-3-kinase (PI3K) to the membrane. The resulting catalytic product, phosphatidylinositol-3,4,5-triphosphate (PIP_3)could, in turn, trigger actin reorganization.[61–66] However, later experiments have proven that novel pathways may be involved in actual sensing mechanism.[67,68] In experiments with neutrophils from chronic granulomatous disease (patients and mice),[69,70] heightened recruitment of neutrophils with multiple lamelipodia to inflammatory stimuli were observed. Failure to produce O_2^{-} results in persistence of cells with depressed bactericidal activity and increased half lives of pro-inflammatory chemokines.

INTEGRINS AND RAFTS AS PLATFORMS FOR FOCAL SIGNALING

Although, the cellular events that transpire during migration have been well documented, the precise biochemistry behind gradient sensing in chemotaxis remains speculative. There is evidence that focal production of ROS at lamellipodia is able to mediate a successful chemotactic event. This is possible due to interactions between the integrin based cell adhesions scaffolds and lipid rafts. The latter are cholesterol and sphinomyelin enriched ordered microdomains present in the cell membrane. These are sites of anchorage of numerous signaling relevant small molecules such as Ras, G-protein coupled receptor, and receptors tyrosine kinases. Caveolae, are flask shaped local invaginations of the plasma membrane[71,72] characterized by the presence of caveolins.[73–76] Caveolin-1 binds to filamin, an actin based protein.[77] Therefore, by retaining caveolin in focal adhesions and regulating their internalization,[25,78] remodeling of the cytoskeleton may be accomplished by mitogens, infective agents, chemical modifiers and mechano-transduction. Evidence for an integrin independent model, too, has been validated.[79]

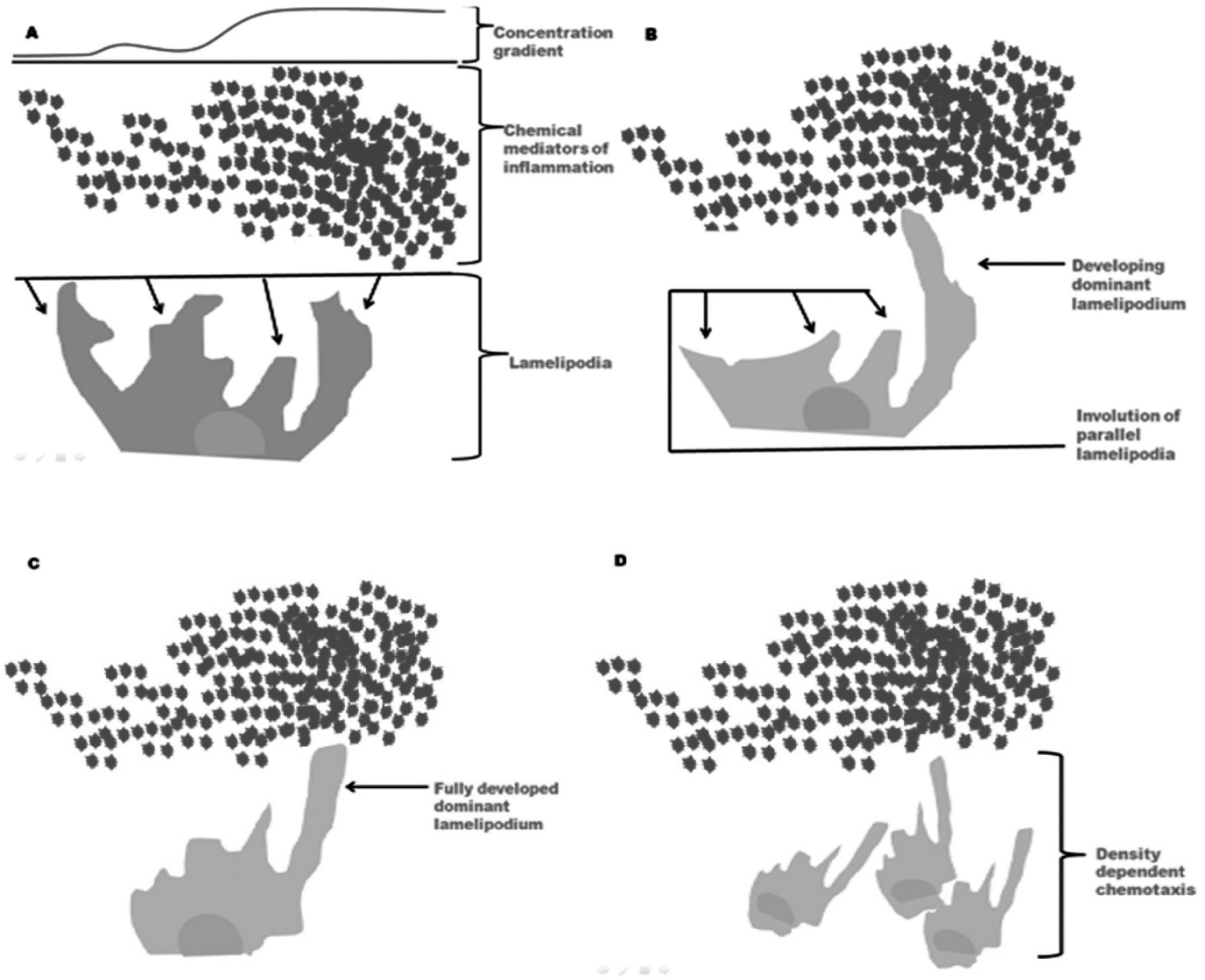

Figure 2 | Gradient sensing in phagocytic competent neutrophils A) Multiple lamelipodia encounters concentration gradient of chemo-attractant, B) Sensing causes appearance of single dominant lamelipodium while, others progressively involute, C) single lamelipodium prevails, D) direction specific transmigration of neutrophils

EXTRACELLULAR SUPEROXIDE DISMUTASE (ECSOD) – FACILITATES VECTORIAL CHEMOTAXIS

The enzyme ECSOD is a large 135 KDa homo-tetrameric protein secreted by smooth muscle cells and macrophages and is bound to the proteoglycan heparin of the extra-cellular matrix.[80,81] This binding ensures a prolonged half life of ~ 85 hours[82] for the enzyme. The ECSOD in humans is encoded on chromosome 4 by the gene *sod3*,[83,84] has an activity of 1 x 10^9 M^{-1} s^{-1}/ Cu,[85] and has a plasma variant.[86,87] ECSOD has a prominent anti-oxidative role in the body, both circulatory and tissue bound. The catalytic activity of the bound form is mediated in part by the interaction with the ECM and disruption of this contact impairs ECSOD dismutase action.[87] Oxidative stress results in down regulation of the enzyme, activation by IFN-γ, depression by TGF-β and intermediate states by TNF-α and IFN-α.[88–91]

A model (Figure 3), for this gradient discerning activity of migrating neutrophils is presented. Briefly, there is competition between intracellular pro-inflammatory molecules (cellular ROS) brought about by damage to tissue (muscle and/or vasculature) by injury and infection, and extra-cellular O_2^- generated by incoming neutrophils, for the ECSOD (extra-cellular superoxide dismutase). This saturates the enzyme and, along with, increased H_2O_2 levels inhibits ECSOD.[92] The peroxide in turn associates with and activates integrin receptors, and is transported via aquaporin and /or ClC3 channels to the subcellular membrane compartment wherein actin rmodification is initiated. These changes include S-glutathionylation,[93,94] nitrosation,[94,95] carbonylation[96,97] and oxidation by disulfide bond formation.[98,99] Indirect effects are mediated through several actin binding proteins. Repeated cycles as discussed above are able to convert the gradient signal into directed chemotaxis involving a dominant lamelipodium. The role of ECSOD derived H_2O_2 in VEGF mediated angiogenesis[100] lends credence to this hypothesis.

CONCLUDING REMARKS

The response of a cell to stimuli may best be described as a 'fuzzy' interplay of several events that results in behavior that is digital or quantal. This is dependent on the nature and location of scaffolding platforms that separate the compartments. These promote cycles of assembly-disassembly locally and serve to determine the final outcome, i.e., 'switch like' in the case of plasma membrane signaling and 'continuous' as in signaling cascades which are endosomal membrane specific, biocatalysis, physiological sensing such as olfaction, vision, long term potentiation and depression (LTP and LTD). In this review, we have discussed the role of neutrophil chemotaxis in a microenvironment of graded inflammatory signals, and the mechanisms of perceiving these at the cellular level. Clusters of integrins in association with lipid rafts are critical

to the development of these distributed sensors which coat the phagocytic competent cell and enable microscopic reaction times. Pertubations of these have been shown to result in inefficient phagocytosis, increased susceptibility to infection, chronic inflammation, progress to malignancy, autoimmune diseases, inappropriate allergies and hypersensitive reactions. The mystery of stimulus directed movement is far from resolved. Unravelling the underlying molecular processes that govern chemotaxis will advance our comprehension of several dependent phenomena in health and disease.

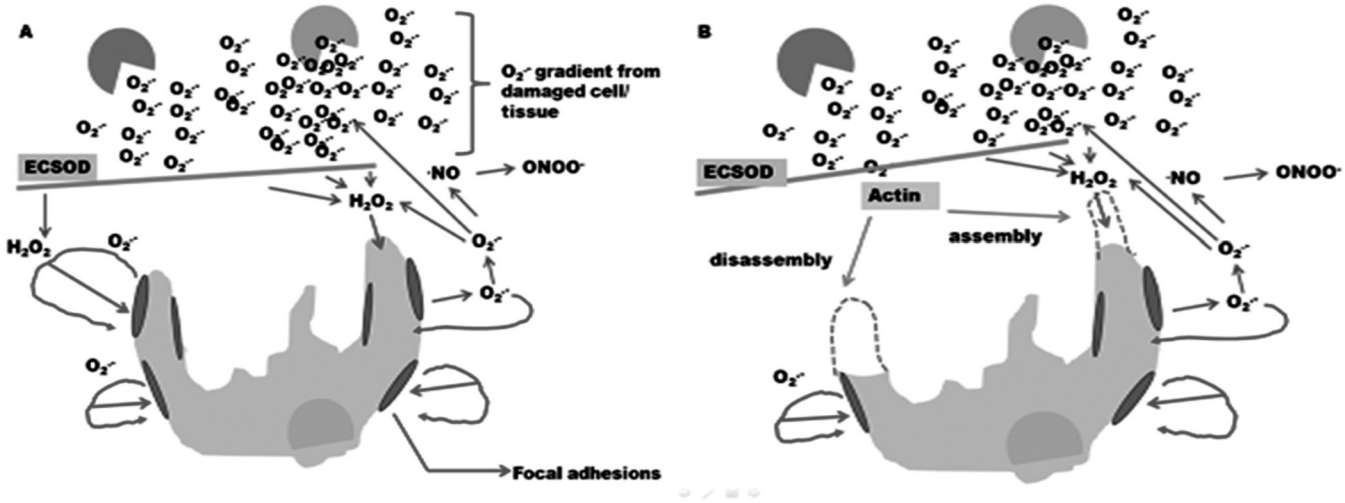

Figure 3 | Model depicting possible role of extra-cellular superoxide dismutase (ECSOD) in gradient sensing. A) Focal adhesions (FAs) are sites of ROS production. Competition between these and intracellular ROS for ECSOD. B) Explosive production of H_2O_2 inactivates ECSOD and triggers actin reorganization leading to development of a dominant lamelipodium.

REFERENCES

1. Berrier AL, Yamada KM. Cell-matrix adhesion. *J Cell Physiol* 2007;213(3): 565–73.
2. Nobes CD, Hall A. Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell* 1995;81:53–62.
3. Allen, WE, Jones GE, Pollard JW, Ridley AJ. Rho, Rac and Cdc42 regulate actin organization and cell adhesion in macrophages. *J Cell Sci* 1997;110:707–720
4. Rottner K, Hall A, Small JV. Interplay between Rac and Rho in the control of substrate contact dynamics. *Curr Biol* 1999;9(12):640–8.
5. Luo BH, Carman CV, Springer TA. Structural basis of integrin regulation and signaling. *Annu Rev Immunol* 2007;25:619–47.
6. Monkley SJ, Zhou XH, Kinston SJ, Giblett SM, Hemmings L, Priddle H, Brown JE, Pritchard CA, Critchley DR, Fassler R. Disruption of the talin gene arrests mouse development at the gastrulation stage. *Dev Dynamic* 2000;219:560–74.
7. Hagel M, George EL, Kim A, Tamimi R, Opitz SL, Turner CE, Imamoto A, Thomas SM. The adaptor protein paxillin is essential for normal development in the mouse and is a critical transducer of fibronectin signaling. *Mol Cell Biol* 2002;22:901–15.
8. Xu W, Baribault H, Adamson ED. Vinculin knockout results in heart and brain defects during embryonic development. *Development* 1998;125:327–37.
9. Pavalko FM, Burridge K. Disruption of the actin cytoskeleton after microinjection of proteolytic fragments of alpha-actinin. *J Cell Biol* 1991;114(3):481–91.
10. Furuta Y, Ilic D, Kanazawa S, Takeda N, Yamamoto T, Aizawa S. Mesodermal defect in late phase of gastrulation by a targeted mutation of focal adhesion kinase, FAK. Oncogene 1995;11:1989–95.
11. Avraham S, Avraham H. Characterization of the novel focal adhesion kinase RAFTK in hematopoietic cells. *Leuk Lymphoma* 1997;27(3-4):247–56.
12. Sakai T, Li S, Docheva D, Grashoff C, Sakai K, Kostka G, Braun A, Pfeifer A, Yurchenco PD, Fassler R. Integrin-linked kinase (ILK) is required for polarizing the epiblast, cell adhesion, and controlling actin accumulation. *Genes Dev* 2003;17:926–40.
13. Zervas CG, Gregory SL, Brown NH. Drosophila integrin-linked kinase is required at sites of integrin adhesion to link the cytoskeleton to the plasma membrane. *J Cell Biol* 2001;152:1007–18.
14. Harden N, Lee J, Loh HY, Ong YM, Tan I, Leung T, Manser E, Lim L. A Drosophila homolog of the Rac- and Cdc42-activated serine/threonine kinase PAK is a potential focal adhesion and focal complex protein that colocalizes with dynamic actin structures. *Mol Cell Biol* 1996;16(5):1896–908.
15. Chen HC, Guan JL. Stimulation of phosphatidylinositol 3'-kinase association with focal adhesion kinase by platelet-derived growth factor. *J Biol Chem* 1994;269(49):31229–33.
16. Tamura M, Gu J, Matsumoto K, Aota S, Parsons R, Yamada KM. Inhibition of cell migration, spreading, and focal adhesions by tumor suppressor PTEN. *Science* 1998;280(5369):1614–7.
17. Angers-Loustau A, Cote JF, Charest A, Dowbenko D, Spencer S, Lasky LA, Tremblay ML. Protein tyrosine phosphatase-PEST regulates focal adhesion disassembly, migration, and cytokinesis in fibroblasts. *J Cell Biol* 1999;144:1019–31.
18. Li S, Bordoy R, Stanchi F, Moser M, Braun A, Kudlacek O, Wewer UM, Yurchenco PD, Fassler R. PINCH1 regulates cell-matrix and cell-cell adhesions, cell polarity and cell survival during the peri-implantation stage. *J Cell Sci* 2005;118:2913–21.
19. Zhang Y, Chen K, Tu Y, Velyvis A, Yang Y, Qin J, Wu C. Assembly of the PINCH-ILK-CH-ILKBP complex precedes and is essential for localization of each component to cell-matrix adhesion sites. *J Cell Sci* 2002;115:4777–86.
20. Ridley AJ, Paterson HF, Johnston CL, Diekmann D, Hall A. The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. *Cell* 1992;70:401–10.
21. Ridley AJ, Hall A. The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell* 1992;70:389–99.
22. Li Z, Hannigan M, Mo Z, Liu B, Lu W, Wu Y, et al. Directional sensing requires Gbetagamma-mediated PAK1 and PIXalpha-dependent activation of Cdc42. *Cell* 2003;114:215–27.
23. Lyons PD, Dunty JM, Schaefer EM, Schaller MD. Inhibition of the catalytic activity of cell adhesion kinase beta by protein-tyrosine phosphatase-PEST-mediated dephosphorylation. *J Biol Chem* 2001;276:24422–31.
24. Sastry, SK, Lyons PD, Schaller MD, Burridge K. PTP-PEST controls motility through regulation of Rac1. *J Cell Sci* 2002;115:4305–16.
25. del Pozo MA, Alderson NB, Kiosses WB, Chiang HH, Anderson RG, Schwartz MA. Integrins regulate Rac targeting by internalization of membrane domains. *Science* 2004;303:839–42.
26. Selvakumar B, Hess DT, Goldschmidt-Clermont PJ, Stamler JS. Co-regulation of constitutive nitric oxide synthases and NADPH oxidase by the small GTPase. *Rac FEBS Lett* 2008;582(15):2195–202.
27. Forman HJ, Fukuto JM, Torres M. Redox signaling: thiol chemistry defines which reactive oxygen and nitrogen species can act as second messengers. *Am J Physiol Cell Physiol* 2004;287(2):C246–56.
28. Torres M, Forman HJ. Redox signaling and the MAP kinase pathways. *Biofactors* 2003;17(1-4):287–96.
29. Duracková Z. Some current insights into oxidative stress. *Physiol Res* 2010;59(4):459–69.
30. Mikkelsen RB, Wardman P. Biological chemistry of reactive oxygen and nitrogen and radiation-induced signal transduction mechanisms. *Oncogene* 2003;22:5734–54.
31. Hawkins BJ, Madesh M, Kirkpatrick CJ, Fisher AB. Superoxide flux in endothelial cells via the chloride channel-3 mediates intracellular signaling. *Mol Biol Cell* 2007;18(6):2002–12.
32. Fisher AB. Redox signaling across cell membranes. *Antioxid Redox Signal* 2009;11(6):1349–56.
33. Mumbengegwi DR, Li Q, Li C, Bear CE, Engelhardt JF. Evidence for a superoxide permeability pathway in endosomal membranes. *Mol Cell Biol* 2008;28:3700–12.

34. Crawford LE, Milliken EE, Irani K, Zweier JL, Becker LC, Johnson TM, et al. Superoxide-mediated actin response in post-hypoxic endothelial cells. *J Biol Chem* 1996;271:26863–7.
35. Nimnual AS, Taylor LJ, Bar-Sagi D. Redox-dependent downregulation of Rho by Rac. *Nat Cell Biol* 2003;5:236–41.
36. Wojciak-Stothard B, Tsang LY, Haworth SG. Rac and Rho play opposing roles in the regulation of hypoxia/reoxygenation-induced permeability changes in pulmonary artery endothelial cells. *Am J Physiol Lung Cell Mol Physiol* 2005;288:L749–60.
37. Moldovan L, Irani K, Moldovan NI, Finkel T, Goldschmidt-Clermont PJ. The actin cytoskeleton reorganization induced by Rac1 requires the production of superoxide. *Antioxid Redox Signal* 1999;1:29–43.
38. Moldovan L, Moldovan NI, Sohn RH, Parikh SA, Goldschmidt-Clermont PJ. Redox changes of cultured endothelial cells and actin dynamics. *Circ Res* 2000;86:549–57.
39. Ushio-Fukai M, Tang Y, Fukai T, Dikalov SI, Ma Y, Fujimoto M, Quinn MT, Pagano PJ, Johnson C, Alexander RW. Novel role of gp91(phox)-containing NAD(P)H oxidase in vascular endothelial growth factor-induced signaling and angiogenesis. *Circ Res* 2002;91:1160–7.
40. Wu RF, Gu Y, Xu YC, Nwariaku FE, Terada LS. Vascular endothelial growth factor causes translocation of p47phox to membrane ruffles through WAVE1. *J Biol Chem* 2003;278:36830–40.
41. Grigoriev I, Borisy G, Vorobjev I. Regulation of microtubule dynamics in 3T3 fibroblasts by Rho family GTPases. *Cell Motil Cytoskeleton* 2006;63:29–40.
42. Noritake J,Watanabe T, Sato K,Wang S, Kaibuchi K. IQGAP1: a key regulator of adhesion and migration. *J Cell Sci* 2005;118:2085–92.
43. Pfannstiel J, Cyrklaff M, Habermann A, Stoeva S, Griffiths G, Shoeman R, et al. Human cofilin forms oligomers exhibiting actin bundling activity. *J Biol Chem* 2001;276:49476–84.
44. Kwiatkowski DJ. Functions of gelsolin: motility, signaling, apoptosis, cancer. *Curr Opin Cell Biol* 1999;11:103–8.
45. Arcaro A. The small GTP-binding protein Rac promotes the dissociation of gelsolin from actin filaments in neutrophils. *J Biol Chem* 1998;273:805–13.
46. Wen D, Corina K, Chow EP, Miller S, Janmey PA, Pepinsky RB. The plasma and cytoplasmic forms of human gelsolin differ in disulfide structure. *Biochemistry* 1996;35:9700–9.
47. Allen PG. Functional consequences of disulfide bond formation in gelsolin. *FEBS Lett* 1997;401:89–94.
48. Tseng Y, An KM, Esue O, Wirtz D. The bimodal role of filamin in controlling the architecture and mechanics of F-actin networks. *J Biol Chem* 2004;279: 1819–26.
49. Hastie LE, Patton WF, Hechtman HB, Shepro D. Filamin redistribution in an endothelial cell reoxygenation injury model. *Free Radic Biol Med* 1997;22: 955–66.
50. Hastie LE, Patton WF, Hechtman HB, Shepro D. Metabolites of the phospholipase D pathway regulate H2O2-induced filamin redistribution in endothelial cells. *J Cell Biochem* 1998;68:511–24.
51. Usatyuk PV, Natarajan V. Regulation of reactive oxygen species induced endothelial cell–cell and cell–matrix contacts by focal adhesion kinase and adherens junction proteins. *Am J Physiol Lung Cell Mol Physiol* 2005;289:L999–L1010.
52. Mitra SK, Hanson DA, Schlaepfer DD. Focal adhesion kinase: in command and control of cell motility. *Nat Rev Mol Cell Biol* 2005;6:56–68.
53. Park SY, Schinkmann KA, Avraham S. RAFTK/Pyk2 mediates LPA-induced PC12 cell migration. *Cell Signal* 2006;18(7):1063–71.
54. Okigaki M, Davis C, Falasca M, Harroch S, Felsenfeld DP, Sheetz MP, Schlessinger J. Pyk2 regulates multiple signaling events crucial for macrophage morphology and migration. *Proc Natl Acad Sci USA* 2003;100:10740–5.
55. Vepa S, Scribner WM, Parinandi NL, English D, Garcia JG, Natarajan V. Hydrogen peroxide stimulates tyrosine phosphorylation of focal adhesion kinase in vascular endothelial cells. *Am J Physiol* 1999;277:L150–8.
56. Gozin A, Franzini E, Andrieu V, Da Costa L, Rollet-Labelle E, Pasquier C. Reactive oxygen species activate focal adhesion kinase, paxillin and p130cas tyrosine phosphorylation in endothelial cells. *Free Radic Biol Med* 1998;25:1021–32.
57. Yang S, Yip R, Polena S, Sharma M, Rao S, Griciene P, et al. Reactive oxygen species increased focal adhesion kinase production in pulmonary microvascular endothelial cells. *Proc West Pharmacol Soc* 2004;47:54–6.
58. Fukata M, Nakagawa M, Kaibuchi K. Roles of Rho-family GTPases in cell polarisation and directional migration. *Curr Opin Cell Biol* 2003;15:590–7.
59. Watanabe T,Wang S, Noritake J, Sato K, Fukata M, Takefuji M, et al. Interaction with IQGAP1 links APC to Rac1, Cdc42, and actin filaments during cell polarization and migration. *Dev Cell* 2004;7:871–83.
60. Tzima E. Role of small GTPases in endothelial cytoskeletal dynamics and the shear stress response. *Circ Res* 2006;98:176–85.
61. Van Keymeulen A, Wong K, Knight ZA, Govaerts C, Hahn KM, Shokat KM, Bourne HR. To stabilize neutrophil polarity, PIP3 and Cdc42 augment RhoA activity at the back as well as signals at the front. *J Cell Biol* 2006;174(3): 437–45.
62. Wong K, Pertz O, Hahn K, Bourne H. Neutrophil polarization: spatiotemporal dynamics of RhoA activity support a self-organizing mechanism. *Proc Natl Acad Sci U S A* 2006;103(10):3639–44.
63. Singleton PA, Dudek SM, Chiang ET, Garcia JG. Regulation of sphingosine 1-phosphate-induced endothelial cytoskeletal rearrangement and barrier enhancement by S1P1 receptor, PI3 kinase, Tiam1/Rac1, and alpha-actinin. *FASEB J* 2005;19(12):1646–56.
64. Sadhu C, Masinovsky B, Dick K, Sowell CG, Staunton DE. Essential role of phosphoinositide 3-kinase delta in neutrophil directional movement. *J Immunol* 2003;170(5):2647–54.
65. Innocenti M, Frittoli E, Ponzanelli I, Falck JR, Brachmann SM, Di Fiore PP, Scita G. Phosphoinositide 3-kinase activates Rac by entering in a complex with Eps8, Abi1, and Sos-1. *J Cell Biol* 2003;160(1):17–23.
66. Zhou K, Pandol S, Bokoch G, Traynor-Kaplan AE. Disruption of Dictyostelium PI3K genes reduces 32Pphosphatidylinositol 3,4 bisphosphate and 32Pphosphatidylinositol trisphosphate levels, alters F-actin distribution and impairs pinocytosis. *J Cell Sci* 1998;111 (Pt 2):283–94.
67. Chodniewicz D, Zhelev DV. Chemoattractant receptor-stimulated F-actin polymerization in the human neutrophil is signaled by 2 distinct pathways. *Blood* 2003;101:1181–4.
68. Andrew N, Insall RH. Chemotaxis in shallow gradients is mediated independently of PtdIns 3-kinase by biased choices between random protrusions. *Nat Cell Biol* 2007;9:193–200.
69. Hattori H, Subramanian KK, Sakai J, Luo HR. Reactive oxygen species as signaling molecules in neutrophil chemotaxis. *Commun Integr Biol* 2010;3(3):278–81.
70. Hattori H, Subramanian KK, Sakai J, Jia Y, Li Y, Porter TF, Loison F, Sarraj B, Kasorn A, Jo H, Blanchard C, Zirkle D, McDonald D, Pai SY, Serhan CN, Luo HR. Small-molecule screen identifies reactive oxygen species as key regulators of neutrophil chemotaxis. *Proc Natl Acad Sci U S A* 2010;107(8):3546–51.
71. Yamada, E. The fine structure of the gall bladder epithelium of the mouse. *J Biophys Biochem Cytol* 1955;1:445–58.
72. Harder T. Simons K. Caveolae, DIGs, and the dynamics of sphingolipid-cholesterol microdomains. *Curr Opin Cell Biol* 1997;9:534–42.
73. Kogo H, Aiba, T, Fujimoto T. Cell type-specific occurrence of caveolin-1alpha and -1beta in the lung caused by expression of distinct mRNAs. *J Biol Chem* 2004;279:25574–81.
74. Rothberg KG, Heuser JE, Donzell WC, Ying YS, Glenney JR, Anderson RG. Caveolin, a protein component of caveolae membrane coats. *Cell* 1992;68: 673–82.
75. Scherer PE, Okamoto T, Chun M, Nishimoto I, Lodish HF, Lisant MP. Identification, sequence, and expression of caveolin-2 defines a caveolin gene family. *Proc Natl Acad Sci USA* 1996;93:131–5.
76. Tang Z, Scherer PE, Okamoto T, Song K, Chu C, Kohtz DS, Nishimoto I, Lodish HF, Lisanti MP. Molecular cloning of caveolin-3, a novel member of the caveolin gene family expressed predominantly in muscle. *J Biol Chem* 1996;271:2255–61.
77. Stahlhut M, van Deurs B. Identification of filamin as a novel ligand for caveolin-1: evidence for the organization of caveolin-1-associated membrane domains by the actin cytoskeleton. *Mol Biol Cell* 2000;11:325–37.
78. Ushio-Fukai M. Compartmentalization of redox signaling through NADPH oxidase-derived ROS. *Antioxid Redox Signal* 2009;11(6):1289–99.
79. Carbo C, Duerschmied D, Goerge T, Hattori H, Sakai J, Cifuni SM, White GC 2nd, Chrzanowska-Wodnicka M, Luo HR, Wagner DD. Integrin-independent role of CalDAG-GEFI in neutrophil chemotaxis. *J Leukoc Biol* 2010;88(2):313–9.
80. Karlsson K, Lindahl U, Marklund SL. Binding of human extracellular superoxide dismutase C to sulphated glycosaminoglycans. *Biochem J* 1988;256:29–33.
81. Adachi T, Yamada H, Yamada Y, Morihara N, Yamazaki N, Murakami T, Futenma A, Kato K, Hirano K. Substitution of glycine for arginine-213 in extracellular-superoxide dismutase impairs affinity for heparin and endothelial cell surface. *Biochem J* 1996;313(Pt 1):235–9.
82. Karlsson K, Sandström J, Edlund A, Marklund SL. Turnover of extracellular-superoxide dismutase in tissues. *Lab Invest* 1994;70(5):705–10.
83. Hjalmarsson K, Marklund SL, Engstrom A, Edlund T. Isolation and sequence of complementary DNA encoding human extracellular superoxide dismutase. *Proc Natl Acad Sci U S A* 1987;84:6340–4.
84. Folz RJ, Crapo JD. Extracellular superoxide dismutase (SOD3): tissue-specific expression, genomic characterization, and computer-assisted sequence analysis of the human EC SOD gene. *Genomics* 1994;22: 162–71.
85. Marklund SL. Human copper-containing superoxide dismutase of high molecular weight. *Proc Natl Acad Sci U S A* 1982;79:7634–8.
86. Marklund SL, Holme E, Hellner L. Superoxide dismutase in extracellular fluids. *Clin Chim Acta* 1982;126:41–51.
87. Sandstrom J, Nilsson P, Karlsson K, Marklund SL. 10-fold increase in human plasma extracellular superoxide dismutase content caused by a mutation in heparin-binding domain. *J Biol Chem* 1994;269:19163–6.
88. Regulation by cytokines of extracellular superoxide dismutase and other superoxide dismutase isoenzymes in fibroblasts. Marklund SL. *J Biol Chem* 1992;267(10):6696–701.
89. Gao F, Koenitzer JR, Tobolewski JM, Jiang D, Liang J, Noble PW, Oury TD. Extracellular superoxide dismutase inhibits inflammation by preventing oxidative fragmentation of hyaluronan. *J Biol Chem* 2008;283:6058–66.

90. Strålin P, Marklund SL. Effects of oxidative stress on expression of extracellular superoxide dismutase, CuZn-superoxide dismutase and Mn-superoxide dismutase in human dermal fibroblasts. *Biochem J* 1994;298 (Pt 2):347–52.
91. Strålin P, Jacobsson H, Marklund SL. Oxidative stress, NO* and smooth muscle cell extracellular superoxide dismutase expression. *Biochim Biophys Acta* 2003;1619(1):1–8.
92. Wedgwood S, Lakshminrusimha S, Fukai T, Russell JA, Schumacker PT, Steinhorn RH. Hydrogen peroxide regulates extracellular superoxide dismutase activity and expression in neonatal pulmonary hypertension. *Antioxid Redox Signal* 2010;Epub: PMID:20919937
93. Dalle-Donne I, Giustarini D, Rossi R, Colombo R, Milzani A. Reversible S-glutathionylation of Cys 374 regulates actin filament formation by inducing structural changes in the actin molecule. *Free Radic Biol Med* 2003;34(1): 23–32.
94. Giustarini D, Milzani A, Aldini G, Carini M, Rossi R, Dalle-Donne I. S-nitrosation versus S-glutathionylation of protein sulfhydryl groups by S-nitrosoglutathione. *Antioxid Redox Signal* 2005;7(7-8):930–9.
95. Yang Y, Loscalzo J. S-nitrosoprotein formation and localization in endothelial cells. *Proc Natl Acad Sci U S A* 2005;102(1):117–22.
96. Dalle-Donne I, Rossi R, Giustarini D, Gagliano N, Lusini L, Milzani A, Di Simplicio P, Colombo R. Actin carbonylation: from a simple marker of protein oxidation to relevant signs of severe functional impairment. *Free Radic Biol Med* 2001;31(9):1075–83.
97. Banan A, Zhang Y, Losurdo J, Keshavarzian A. Carbonylation and disassembly of the F-actin cytoskeleton in oxidant induced barrier dysfunction and its prevention by epidermal growth factor and transforming growth factor alpha in a human colonic cell line. *Gut* 2000;46(6):830–7.
98. DalleDonne I, Milzani A, Colombo R. H2O2-treated actin: assembly and polymerinteractions with cross-linking proteins. *Biophys J* 1995;69(6):2710–9.
99. Milzani A, DalleDonne I, Colombo R. Prolonged oxidative stress on actin. *Arch Biochem Biophys* 1997;339(2):267–74.
100. Oshikawa J, Urao N, Kim HW, Kaplan N, Razvi M, McKinney R, Poole LB, Fukai T, Ushio-Fukai M. Extracellular SOD-derived H2O2 promotes VEGF signaling in caveolae/lipid rafts and post-ischemic angiogenesis in mice. *PLoS One* 2010;5(4):e10189:PMID:20976168.