The Zinc Sensing Receptor, a Link Between Zinc and Cell Signaling

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Zinc is essential for cell growth. For many years it has been used to treat various epithelial disorders, ranging from wound healing to diarrhea and ulcerative colon disease. The physiological/molecular mechanisms linking zinc and cell growth, however, are not well understood. In recent years, Zn^{2+} has emerged as an important signaling molecule, activating intracellular pathways and regulating cell fate. We have functionally identified an extracellular zinc sensing receptor, called zinc sensing receptor (ZnR), that is specifically activated by extracellular Zn^{2+} at physiological concentrations. The putative ZnR is pharmacologically coupled to a Gq-protein which triggers release of Ca^{2+} from intracellular stores via the Inositol 1,4,5–trisphosphate (IP_3) pathway. This, in turn results in downstream signaling via the MAP and phosphatydilinositol 3–kinase (PI3 kinase) pathways that are linked to cell proliferation. In some cell types, e.g., colonocytes, ZnR activity also upregulates Na^+/H^+ exchange, mediated by Na^+/H^+ exchanger isoform 1 (NHE1), which is involved in cellular ion homeostasis in addition to cell proliferation. Our overall hypothesis, as discussed below, is that a ZnR, found in organs where dynamic zinc homeostasis is observed, enables extracellular Zn^{2+} to trigger intracellular signaling pathways regulating key cell functions. These include cell proliferation and survival, vectorial ion transport and hormone secretion. Finally, we suggest that ZnR activity found in colonocytes is well positioned to attenuate erosion of the epithelial lining of the colon, thereby preventing or ameliorating diarrhea, but, by signaling through the same pathways, a ZnR may enhance tumor progression in neoplastic disease.

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INTRODUCTION

The zinc gradient across the plasma membrane may reach six orders of magnitude, as cytoplasmic Zn^{2+} is tightly buffered by a variety of mechanisms (1). Specific binding sites for Zn^{2+} are present on numerous proteins, including Zn^{2+} fingers on transcription factors, that bind it with high affinity, and metallothioneins, to which the Zn^{2+} is more loosely bound. Dynamic changes in extracellular Zn^{2+} occur upon its release from cells in organs such as pancreas, brain, and salivary gland (2–4), while changes in intracellular Zn^{2+} may result from oxidative stress (5,6). Such dynamic changes in Zn^{2+} gradients and the availability of specific Zn^{2+} binding domains suggest that Zn^{2+} ions, once considered merely structural elements, are, in fact, important signaling molecules that influence many aspects of cell physiology.

The signaling effects of Zn^{2+} may be mediated by intracellular or extracellular Zn^{2+} ions. It is generally accepted that an increase in free intracellular Zn^{2+} is associated with cell death. For example, release of intracellular Zn^{2+}, triggered by formation of reactive oxygen species (ROS) or by nitrosilation, induces proapoptotic molecules, e.g., p38, and activation of K^+- channels leading to cell death (7,8). Chelation of intracellular Zn^{2+} then, using a high affinity Zn^{2+} chelator, could interfere with this process. Chelation of intracellular Zn^{2+}, however, may remove zinc from intracellular metalloproteins, resulting in protein synthesis-dependent, caspase-3 mediated apoptosis (9).

To protect cells from the consequences of a decrease in cellular zinc, changes in plasma Zn^{2+} often precede the reduction of this ion within cells. Symptoms of zinc deficiency, therefore, particularly attenuation of cell proliferation, are observed long before changes in intracellular zinc are observed (10). Thus, sensing changes in extracellular Zn^{2+}, and activation of signaling to regulate cell processes, before key components (e.g., zinc finger proteins) are affected, is essential. This Zn^{2+}-sensing function might also provide protection against a sudden rise in intracellular Zn^{2+} in tissues in which Zn^{2+} is released during normal activity, e.g., in endocrine and exocrine glands or at glutamatergic synapses in the mammalian forebrain.

Extracellular Zn^{2+} indirectly activates cell signaling, by interacting with major membrane transporters and ion channels, most notably, the dopamine transporter, NMDA, glycine, GABA, and purinergic receptors (11–15). A high affinity binding site has also been

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Table 1: Summary of currently known cells which exhibit Zn^{2+} -dependent Ca^{2+} signaling via the putative ZnR pathway.

	Zn^{2+} -dependent Ca^{2+} rise, via the IP_3 pathway	Signaling pathway activated by extracellular Zn^{2+}	Physiological outcome	Ref
HT29 colonocytes	+ + +	MAPK; PI3K; CaMK; clusterin	NHE1 upregulation	(22,29)
PC-3 androgen independent prostate cancer	+ +	MAPK; PI3K; PKC	Cell proliferation and survival	In prep.
HaCaT, (Normal human) keratinocytes	+ +	MAPK; PI3K	Enhanced wound healing	(22)
3T3 and human Fibroblasts	No response	None	—	
Neurons – acute slice	+ +	MAPK	Unknown	In prep.
Glia cell - primary	No response	None	—	
C6 glioma cells	No response	None	—	
Pancreatic β cells	+ +	Unknown	Unknown	
INS pancreatic α cells	No response	None	—	
HSY salivary gland	+ + +	Unknown	Paracrine activity; ATP secretion	(24)

identified recently on the store-operated channel (SOC) (16).

A growing body of evidence suggests that extracellular Zn^{2+} also activates signal transduction pathways that induce cell proliferation and survival. Thus, Zn^{2+} upregulates the PI3 kinase pathway, leading to activation of AKT in fibroblasts (17). Zinc also has been shown to induce transactivation of the epidermal growth factor receptor (EGFR) by Src in airway epithelial cells (18–20). In colonocytes, extracellular Zn^{2+} triggered the activation of ERK1/2 (extracellular-signal regulated kinase), which, in turn, was accompanied by induction of p21(CiP/WAF1) and cyclin D1 (21). Evidence of a direct mechanism linking extracellular Zn^{2+} to cellular signaling, however, has been lacking. We hypothesized that such a mechanism exists and subsequently described the interaction of extracellular Zn^{2+} with a specific target, an extracellular Zn^{2+} sensing receptor, that mediates Zn^{2+}-dependent intracellular signaling (22).

IDENTIFICATION OF A PUTATIVE Zn^{2+} SENSING RECEPTOR

Because of the synergistic effect of Zn^{2+} and Ca^{2+} on cell growth, and the general role of Ca^{2+} in regulating many of the signaling pathways known to be activated by zinc, we first asked if Zn^{2+}-dependent signaling may be mediated by changes in intracellular Ca^{2+}. Extracellular Zn^{2+} in colonocytes, keratinocytes, and salivary gland cells, was subsequently shown to trigger a rise in intracellular Ca^{2+} released from thapsigargin-sensitive stores (22,23) (Table 1). Inhibitors of Gαq and the phospholipase C, (PLC), attenuated this Zn^{2+}-dependent Ca^{2+} rise, indicating that it is mediated by activation of a Gαq-coupled receptor (22,24). However, PLC could also be activated by a mechanism that involves, among others, receptor tyrosine kinases (23). To determine the possible role of growth factor receptor in mediating the Zn^{2+}-dependent Ca^{2+} response cells were treated with the general inhibitor of tyrosine kinases, genistein (50μM), prior to application of Zn^{2+}. The Zn^{2+} dependent Ca^{2+} rise was not attenuated by the inhibitor indicating it is not mediated by receptor tyrosine kinases (Figure 1A). This activity proved highly specific to extracellular Zn^{2+} as it was not triggered by other heavy metal ions tested, e.g. Mn^{2+}, Cu^{2+}, and Fe^{2+} (22). This is in agreement with previous works showing that cation sensing receptors are not activated by Zn^{2+} (25). Interestingly, a putative Cd^{2+}-sensing receptor was described in fibroblasts (26,27), Zn^{2+} did not activate the Ca^{2+} release mediated via the IP_3 pathway in these cells, similar to our results (Table 1). Although Zn^{2+} did not activate this putative metal receptor, it acted as a competitive inhibitor to Cd^{2+}. Finally, the Zn^{2+}-dependent Ca^{2+} rise is distinct from the activity of the Ca^{2+} sensing receptor (CaR) (28), as overexpression of CaR did not produce an increase in Ca^{2+} following application of extracellular Zn^{2+}. Thus, our experiments revealed a Gq-coupled, Zn^{2+} sensing receptor, ZnR, linking changes in extracellular Zn^{2+} and downstream signal transduction pathways (Figure 1B, 22). Although the molecular identity of the Zn^{2+} sensing receptor has not yet been determined, our preliminary data indicate that ZnR activity is mediated by hetero-oligomerization of two members of the venous-fly trap subgroup of G-protein coupled receptors, which form a functional zinc sensing receptor. Such heterodimerization, plays a role in the versatility of the taste sensing receptors, it remains to be elucidated if it also plays a role in cation sensing.

EXTRACELLULAR Zn^{2+}-DEPENDENT SIGNALING IN THE COLON

In colonocytes, the putative ZnR mediates Zn^{2+}-dependent activation of the MAP kinase and the PI3 kinase pathways (29). Desensitization of the ZnR by Zn^{2+}, is followed by inhibition of the Zn^{2+}-dependent Ca^{2+} rise as well as by phosphorylation of ERK1/2, indicating that the ZnR is a principal link between extracellular Zn^{2+} and ERK1/2. Activation of the ZnR signaling pathway also upregulates the Na^+/H^+ exchanger isoform 1 (NHE1)

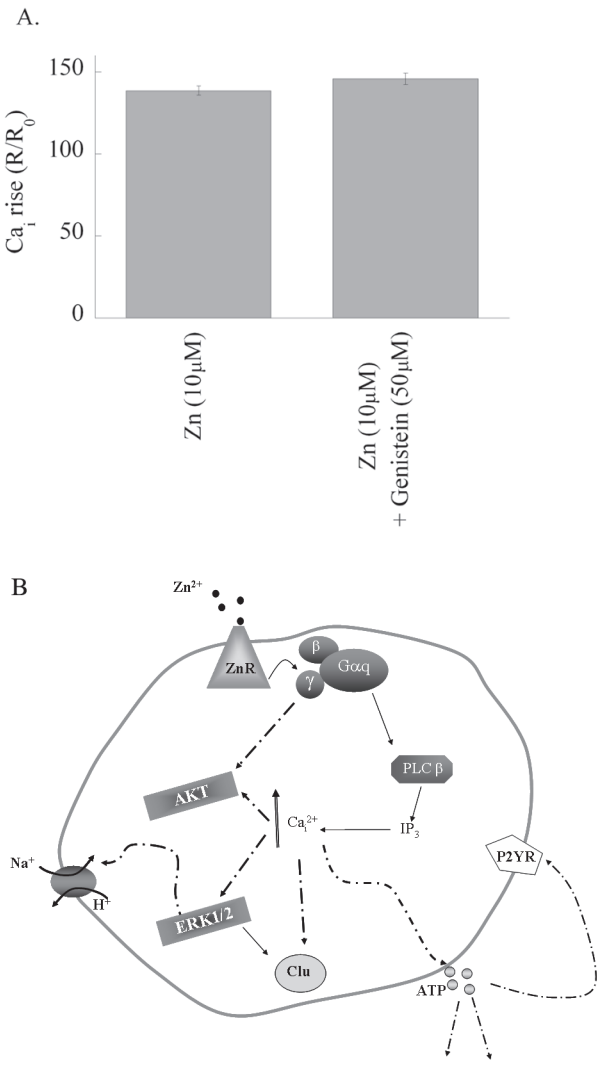

Figure 1. A. The Zn^{2+}–dependent Ca^{2+} rise triggered in HaCaT, keratinocytic cell line, is insensitive to the general tyrosine kinase inhibitor genistein. B. A model for ZnR signaling mediating Zn^{2+}–dependent cell growth and survival. Our results indicate that a ZnR is the major link between changes in extracellular Zn^{2+} and physiological cell function, such as: secretion, proliferation and survival. Desensitization, induced by Zn^{2+}, largely attenuates the Zn^{2+}–dependent signaling.

and enhances the recovery from acidic pH (29). Once thought to merely regulate pHi, this Na^+/H^+ exchanger is now believed to be involved in a much broader range of activities, particularly in regulation of cell proliferation and apoptosis (30–32). Butyrate, a short chain fatty acid produced by bacterial fermentation in the colon, induces apoptosis of colonocytes and has been implicated in ulcerative colon diseases (33–35). Interestingly, it has been demonstrated that zinc confers protection in ulcerative colitis, though the mechanism is not known (36). Upregulation of NHE1 by ZnR activity and subsequent anti-apoptotic and proliferative signaling may explain how zinc helps prevent ulcerative colitis. We have shown that application of extracellular Zn^{2+} prior to acidification with butyrate significantly enhances recovery of the colonocytes from the acidification produced by butyrate. Furthermore, short exposure of colonocytes to concentrations of Zn^{2+} sufficient to activate but not desensitize the ZnR, or to induce changes in intracellular Zn^{2+}, attenuated butyrate-induced cell death (Hershfinkel, et al., In preparation and Figure 2). That this effect was not mediated by regulation of pH_i is shown by the fact that inhibition of Na^+/H^+ exchange, using the NHE1 inhibitor, cariporide, did not alter colon cell survival. This indicates that an extracellular Zn^{2+}-dependnet mechanism can reduce the acid load by activating the NHE1, but must activate an additional pathway to mediate Zn^{2+}-dependent cell survival. One candidate is the pro-survival glycoprotein, clusterin (CLU) (also known as apolipoprotein J) (37). Initial experiments have shown that activation of the ZnR signaling pathway leads to enhancement of clusterin expression. The level of expression, moreover, was enhanced further by application of zinc and butyrate, suggesting a synergistic mechanism. Considering the strong pro-survival effects of clusterin described in the colon (37,8), it is tempting to speculate that such a mechanism may underlie the protective effect of zinc in preventing butyrate-induced cell death. This may further suggest a role for ZnR in the etiology of colon cancer.

A PARACRINE ROLE FOR THE PUTATIVE ZnR

Paracrine effects of signaling molecules trigger intracellular signaling pathways in neighboring cells that do not express a specific receptor. We posit that paracrine effects are of particular importance for amplifying signaling mediated by endogenous zinc, especially in the skin where zinc is involved in wound healing via an unknown mechanism. Yet, zinc is released only in minute amounts following the infliction of a wound or other epithelial damage (39). The release of intracellular Ca^{2+}, such as that induced by the pathway triggered by ZnR activity, is a principle mediator of paracrine responses, and has been shown to induce the release of important agonists such as ATP

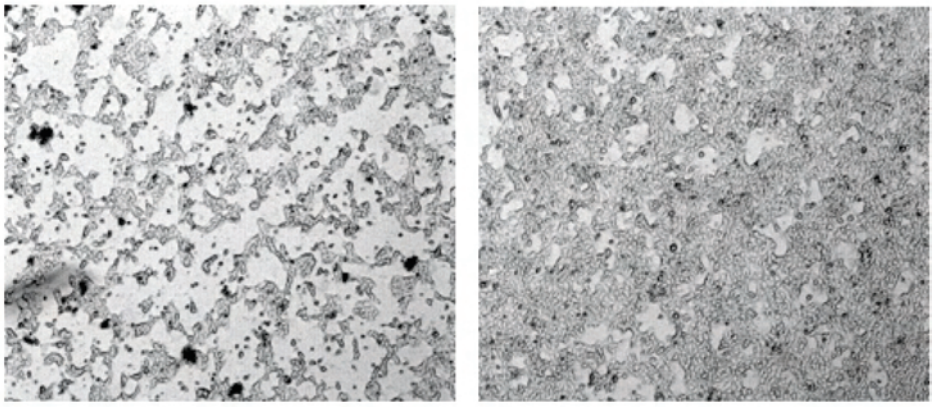

Figure 2. Extracellular Zn^{2+} –signaling, activated by the putative ZnR, reduces butyrate induced cell death. Colonocytic culture, HT29 cells, was treated with butyrate (30 mM, 24 h) and cells were imaged using brightfield microscopy. Massive loss of cells is observed (left picture) following the butyrate treatment. Application of Zn^{2+} for ten minutes prior to the butyrate treatment reduced the loss of cells in the culture (right picture).

(40–42). In addition, it has been demonstrated previously that Zn^{2+} regulates ATP-sensitive purinergic receptor stimulation in a Ca^{2+}-dependent manner (43).

Zinc is packaged in secretory granules of many exocrine glands, such as the salivary glands, and the pancreas (1). Although a specific role for salivary Zn^{2+} is unknown, loss of taste and salivary secretion dysfunctions have been linked to zinc deficiency, suggesting that this ion plays an important role in salivary secretion (44,45). We have observed that Zn^{2+}, acting via the ZnR pathway, mediates release of intracellular Ca^{2+} in the HSY, ductal salivary gland cell line (parotid origin) (24). The endogenous release of ATP and, thereby, activation of the P2Y purinergic, receptor is important for regulation of the ionic content, volume and pH of the salivary fluid (24). We therefore studied the role of extracellular Zn^{2+} in regulating ATP secretion in co-cultures of HSY and vascular smooth muscle cells that do not express a functional ZnR. Activation of the salivary-ZnR in HSY cells was followed by a rise in Ca^{2+} in the vascular cells that was inhibited by the ATP scavenger, apyrase. Our results, therefore, indicate that a salivary-ZnR also acts paracrinically, i.e., by enhancing secretion of ATP, thereby linking zinc and the ZnR activity to key signaling pathways involved in salivary secretion (24). Clearly this model may encompass other secreted molecules, and may, thereby, extend the ability of ZnR to activate intracellular signaling. It may be particularly relevant with regard to the role of ZnR in wound healing. Although zinc is well known to enhance wound healing, we have identified ZnR-dependent signaling on keratinocytes and not on the fibroblasts that play such an important role in this process (22). A paracrine effect could explain how keratinocytic ZnR is initially activated and subsequently induces ATP secretion. The secreted ATP then serves to trigger crosstalk between the keratinocytes and fibroblasts, mediated by the purinergic P2Y receptor. In fact, we observed that producing a scratch in a keratinocytic monolayer, triggers a Zn^{2+}-dependent Ca^{2+} rise (Sharir et al., In preparation), this may then initiate a signaling cascade leading to proliferation of fibroblasts as well.

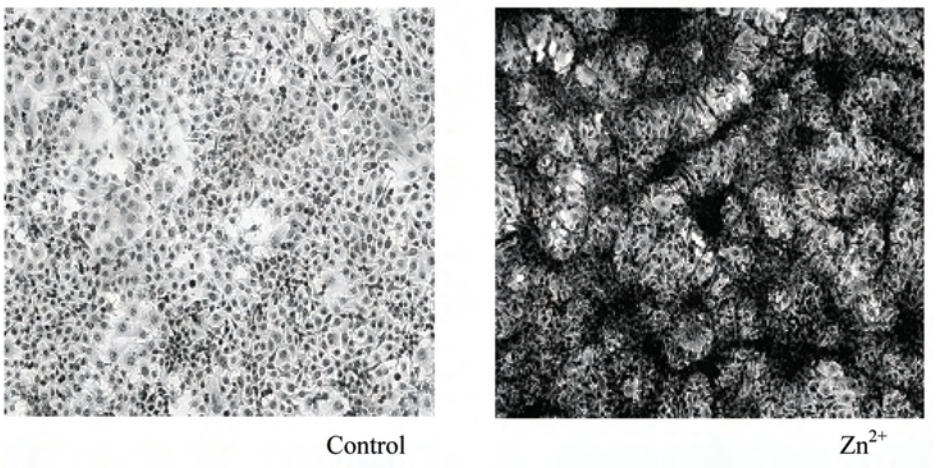

Figure 3. Extracellular Zn^{2+} –signaling, activated by the putative ZnR, enhanced cell growth of prostate cancer cells. Prostate cancer cell line, PC–3, was treated with Zn^{2+} –containing (100 μM, ten minutes) or Zn^{2+} –free Ringer's solution daily, for five days. Cultures were then stained with crystal violet. Enhanced cell growth is shown in the cells treated with Zn^{2+} (right picture) compared with the control cells (left picture).

EXTRACELLULAR Zn^{2+} DEPENDENT SIGNALING IN THE PROSTATE

Robust changes in extracellular Zn^{2+} are observed in the prostate during tumorigenesis (46,47). In the normal, non-neoplastic prostate, Zn^{2+} is found at very high, i.e., millimolar concentrations. It is involved in metabolic functions that result in secretion of citrate which binds the Zn^{2+} (48,49). In prostate cancer, a ten-fold decrease in Zn^{2+} and citrate is observed (50). Yet, cellular signaling, activated by such changes in Zn^{2+}, that affect cell growth and survival, have not been described in the prostate. We have shown that an androgen-independent prostate cancer cell line, PC-3, mediates Zn^{2+}-dependent intracellular Ca^{2+} signals similar to the putative ZnR (Dubi et al., In preparation). The characteristic intracellular Ca^{2+} release mediated by such

ZnR, and the subsequent activation of MAP and PI3 kinases, may enhance prostate cell proliferation and survival. Using crystal violet staining we show that application of Zn^{2+} (for a short ten minutes daily), that activates the ZnR-dependent intracellular signaling but does not significantly change intracellular Zn^{2+}, enhances cell proliferation (Figure 3). The profound reduction in Zn^{2+} during prostate cancer, however, may suggest that ZnR activity, inducing cell growth will be attenuated in prostate cancer. This apparent paradox may be reconciled by our demonstration that the epithelial ZnR is completely desensitized by high Zn^{2+} concentrations such as found in the normal prostate (29). We propose, therefore, that the ZnR activity is quiescent in the non-neoplastic prostate. The reduced Zn^{2+} concentrations occurring in the neoplastic prostate are not inducing desensitization of the ZnR, and thus Zn^{2+}-dependent signaling may be activated leading to cell growth. During prostate cancer, ZnR signaling can be triggered by Zn^{2+} which is released from cells following the tissue destruction triggered by the invasion of the tumor to enhance cell proliferation and survival. Further *in vivo* studies using Zn^{2+} chelation should clarify the novel role of Zn^{2+}-dependent signaling activation in prostate cancer progression.

CONCLUSIONS

Cell signaling triggered by the putative ZnR, and its profound role in mediating Zn^{2+}-dependent signaling, supports our hypothesis that such a receptor is a major link between extracellular zinc and its physiological functions such as secretion, or cell proliferation, and survival (see Figure 1). The ZnR, as a putative member of the G-protein coupled receptor (GPCR) family, which represents two percent of the human genome but is a target for about 50 percent of current pharmaceutical compounds, is particularly attractive as a target candidate for therapeutic interventions (51). At the very least, regulation of ZnR activation is likely to be of significant importance in wound healing, diarrhea, and salivary dysfunction. In each of these conditions, zinc is already used therapeutically, though development of more potent ligands to activate such a ZnR may prove beneficial. The simplicity of Zn^{2+} as a ligand holds out the promise that effective zinc mimetics can be developed. Inhibiting the function of a ZnR, either by reducing ligand availability or by means of its desensitization, also may provide a novel and efficacious strategy for attenuating tumor progression. The availability of effective *in vivo* zinc chelators and carriers, e.g., clioquinol, may, moreover, suggest an effective approach toward this goal.

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