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Report

The Deficiency of PIP_2 5-Phosphatase in Lowe Syndrome Affects Actin Polymerization

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Lowe syndrome is a rare X-linked disorder characterized by bilateral congenital cataracts, renal Fanconi syndrome, and mental retardation. Lowe syndrome results from mutations in the *OCRL1* gene, which encodes a phosphatidylinositol 4,5 bisphosphate 5-phosphatase located in the trans-Golgi network. As a first step in identifying the link between ocrl1 deficiency and the clinical disorder, we have identified a reproducible cellular abnormality of the actin cytoskeleton in fibroblasts from patients with Lowe syndrome. The cellular abnormality is characterized by a decrease in long actin stress fibers, enhanced sensitivity to actin depolymerizing agents, and an increase in punctate F-actin staining in a distinctly anomalous distribution in the center of the cell. We also demonstrate an abnormal distribution of two actin-binding proteins, gelsolin and α-actinin, proteins regulated by both PIP_2 and Ca^{+2} that would be expected to be altered in Lowe cells. Actin polymerization plays a key role in the formation, maintenance, and proper function of tight junctions and adherens junctions, which have been demonstrated to be critical in renal proximal tubule function, and in the differentiation of the lens. These findings point to a general mechanism to explain how this PIP_2 5-phosphatase deficiency might produce the Lowe syndrome phenotype.

The oculocerebrorenal syndrome of Lowe (OCRL [MIM 309000]) is a rare X-linked disorder caused by the deficiency of a PIP_2 5-phosphatase, ocrl1 (Suchy et al. 1995; Zhang et al. 1995). The discovery of the function of ocrl1 and its subcellular localization to the Golgi (Olivos-Glander et al. 1995; Dressman et al. 2000) has not revealed how the enzyme defect leads to the bilateral congenital cataracts, mental retardation, and renal Fanconi syndrome that characterize the disorder. Lowe cells have an elevated concentration of the substrate for ocrl1, PIP_2 (Zhang et al. 1998). PIP_2 is a phospholipid with a pivotal role in a number of fundamental cell processes, including cell signaling, protein trafficking, and polymerization of the actin cytoskeleton (Berridge and Irvine 1989; Toker 1996; Raucher et al. 2000). We describe here our initial work demonstrating the novel finding that Lowe fibroblasts have abnormalities in the actin cytoskeleton, including decreased actin stress fibers and altered response to depolymerizing agents. In addition, we report the abnormal distribution of several actin-binding proteins that require PIP_2 for optimal activity. Because of the importance of actin in cell-cell contacts, we expect that this cellular phenotype will help to identify a mechanism for the occurrence of the Lowe syndrome phenotype.

For these studies, we used fibroblast cultures from patients with Lowe syndrome and one control culture (PHL336) that were obtained with informed consent of a parent or guardian (IRB protocol HG0008A and the corresponding committee at Baylor College of Medicine, as described elsewhere [Lin et al. 1997*b*]). PIP_2 phosphatase activities have been measured on all patients and are <10% of normal activity. Mutations in cell lines were described elsewhere: Lowe line 1 (XL53-01), line 2 (XL49-22), line 4 (LS60), and line 5 (PHL255) (Lin et al. 1997*b;* Gropman et al. 2000); and Lowe line 6 (PHL467) and line 7 (PHL254) (Lin et al. 1998). Lowe line 3 (PHL243) was from a patient with no detectable OCRL1 mRNA and a deficient PIP_2 5-phosphatase activity, but the mutation has not yet been identified (authors' unpublished data). Normal cell lines were obtained from the ATCC, cell line numbers CRL 1509, CRL 1513, and CRL 1489. Cells were cultured at 37°C

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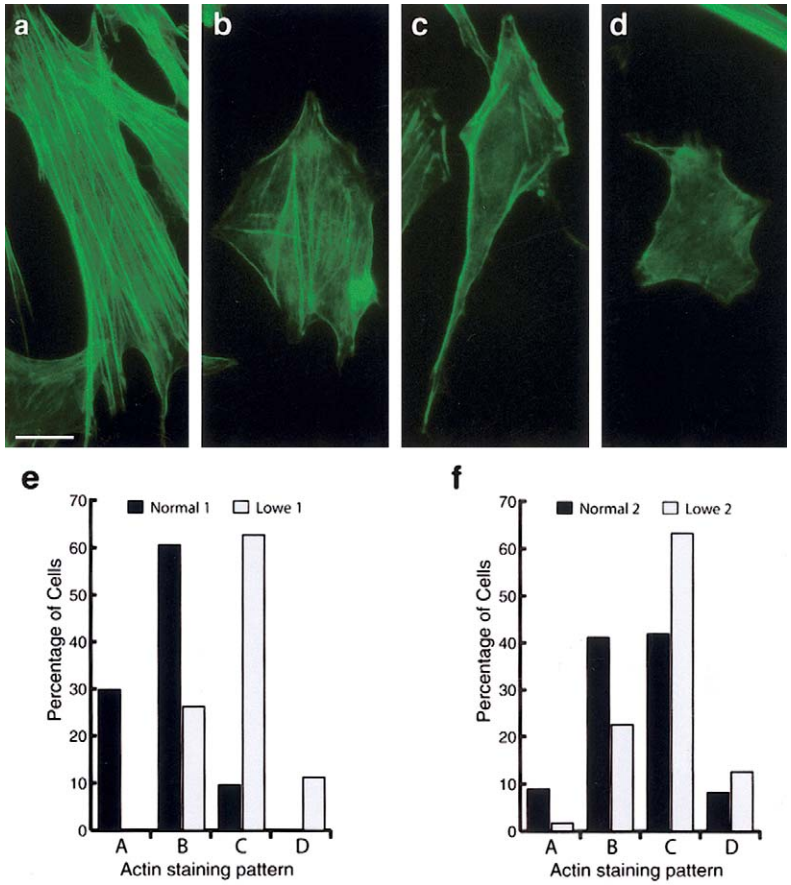

Figure 1 Actin stress-fiber staining is reduced in Lowe fibroblasts. F-actin staining was assessed in Lowe and normal fibroblasts, using Alexa 488 conjugated phalloidin to detect F-actin. Stained cells were classified into one of four types of actin staining: examples of each type (*a–d*), are shown in normal cells. Scale bar = 0.016 μm. The histograms (*e* and *f*) show the results of two experiments with different Lowe and normal fibroblast lines, in which 100 (*e*) and 220 cells (*f*) of each type were scored by an observer masked to the genotype. Lowe syndrome fibroblasts had fewer cells with type a and b staining patterns and more cells with type c and d staining patterns, as compared with control fibroblasts ($\chi^2 = 38.00$, df 7, $P < 10^{-11}$). These results demonstrate that Lowe fibroblasts have a reduced number of long actin stress fibers.

in Dulbecco's modified Eagle medium with 10% fetal calf serum.

We first assessed the actin stress-fiber composition in Lowe and normal human fibroblasts by immunofluorescence with phalloidin, which binds to filamentous actin (F-actin). Cells were grown on eight-well chamber slides (NalgeneNunc) for 48 h, washed with PBS, fixed and permeabilized with 4% buffered paraformaldehyde and 0.1% Triton X-100 for 5 min, blocked with 5% goat serum (NGS) for 30 min, incubated with Alexa 488 conjugated phalloidin (Molecular Probes) in PBS/1% NGS for 1 h, mounted with Fluormount (ICN), and photographed with a Leica epifluorescence microscope with an Optronics MagnaFire CCD camera. To quantitate the staining, we scored >100 cells of each cell line for actin stress-fiber staining using a method reported elsewhere (Verderame et al. 1980). This method classifies F-actin staining into one of four patterns—a, b, c, and d—on the basis of the number and length of actin stress fibers: type a cells have longer, more densely packed actin stress fibers, and type d have no detectable stress fibers. Figure 1*a*–1*d* shows representative normal cells for each pattern. The results of the scoring, performed blind to the genotype, in two experiments with different Lowe and normal fibroblast lines, are shown in figure 1*e* and figure 1*f*. Lowe fibroblasts (lines 2 and 3) had an overall decrease in actin stress-fiber staining with thinner and shorter actin stress fibers that were less tightly packed. The difference between the actin-staining patterns in normal and Lowe cells was significant by χ^2 analysis, with Lowe cells having fewer type a and b staining patterns and more type c and d patterns ($\chi^2 = 38.00$, df 7, $P < 10^{-11}$).

In addition to the reduced stress-fiber staining, Lowe fibroblasts had punctate F-actin staining (fig. 2*a*), present in 64% of cells, whereas only 1% of normal fibroblasts

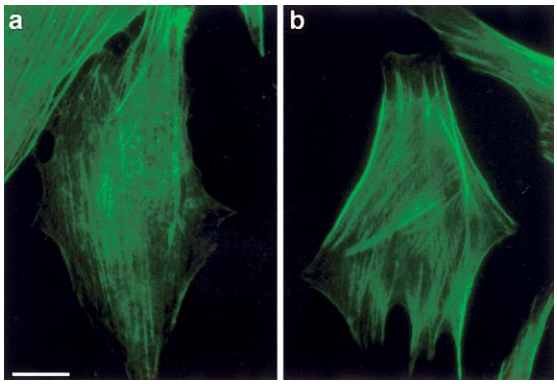

Figure 2 The F-actin staining pattern of Lowe cells shows a punctate pattern in the center of the cell, essentially absent from control cells. Lowe (*a*) and normal (*b*) fibroblasts were stained for F-actin using a fluorescent conjugated phalloidin, as described in figure 1. Scale bar = 0.02 μm.

had such punctate staining (fig. 2*b*). This punctate F-actin staining was more concentrated around the nucleus, as confirmed by staining with 4′,6-diamidino-2-phenylindole (DAPI) (data not shown). Since phalloidin binds to F-actin and not to actin monomer, the punctate staining is due to an increase in short-actin filaments in this region.

So that the abnormal actin stress-fiber staining in the Lowe cells could be better understood, normal fibroblasts and two different Lowe fibroblast cultures were treated with the actin depolymerizing agents cytochalasin D and latrunculin A. These agents depolymerize actin primarily by blocking the addition of actin monomers to the ends of actin filaments. Depolymerization results from a net loss of monomers from filament ends; thus the rate of depolymerization is related to the number of filament ends. Fibroblasts were treated with latrunculin A (Molecular Probes) (.24 μmol) and cytochalasin D (1 μmol) (Sigma) for 5–30 min at 37°C, were washed with PBS, and were stained for F-actin as described above. Higher concentrations of these agents were also used, but they resulted in extreme depolymerization of actin filaments such that intact cells were not detectable by immunofluorescence following fixa-

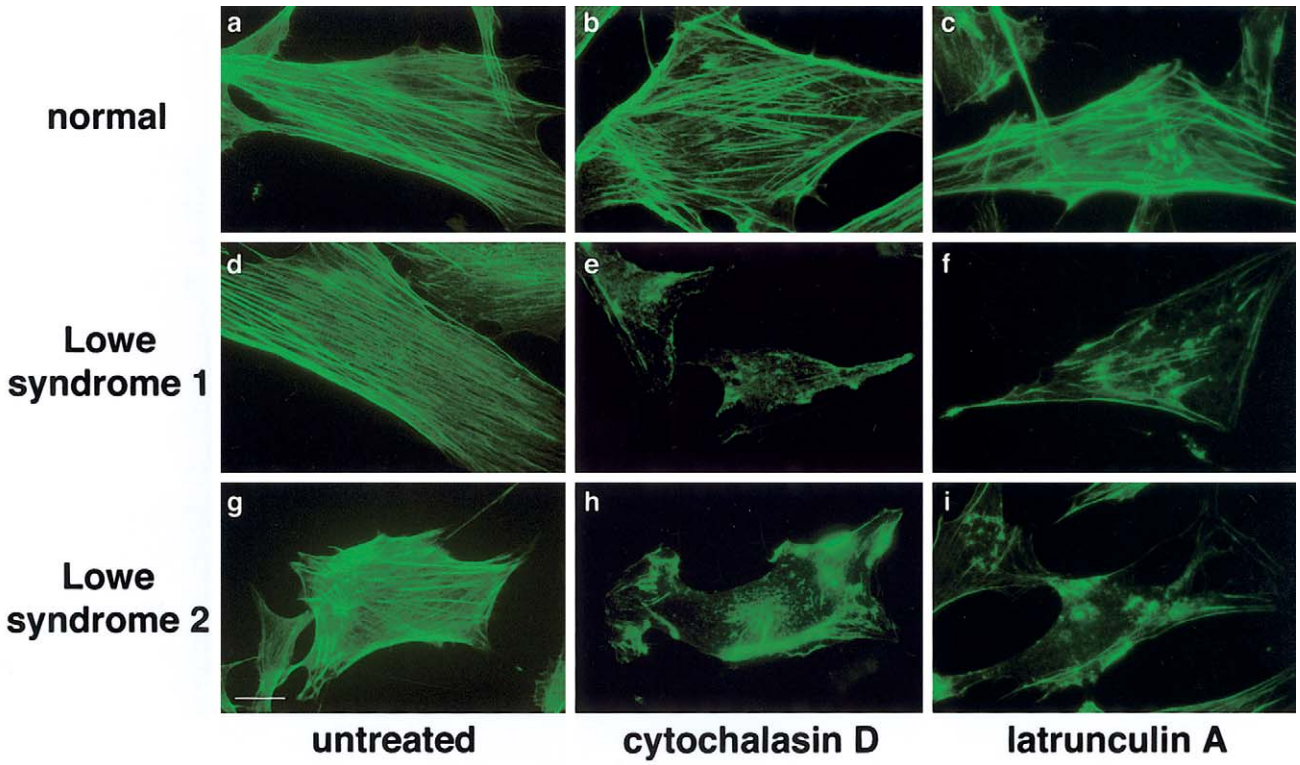

Figure 3 Lowe fibroblasts had a more rapid response than controls to the actin depolymerizing agents, cytochalasin D and latrunculin A. Normal (*a–c*) and two Lowe fibroblast lines (*d–f* and *g–i*) were stained for F-actin using fluorescent conjugated phalloidin, as described in figure 1. Fibroblasts are shown untreated (*a*, *d*, and *g*) or incubated 5 min with 1 μmol cytochalasin D (*b*, *e*, and *h*) or with 0.24 μmol latrunculin A (*c*, *f*, and *i*). Lowe cells (*e*, *f*, *h*, and *i*) showed a greater response to depolymerizing agents than did control cells (*b* and *c*). Scale bar = 0.02 μm.

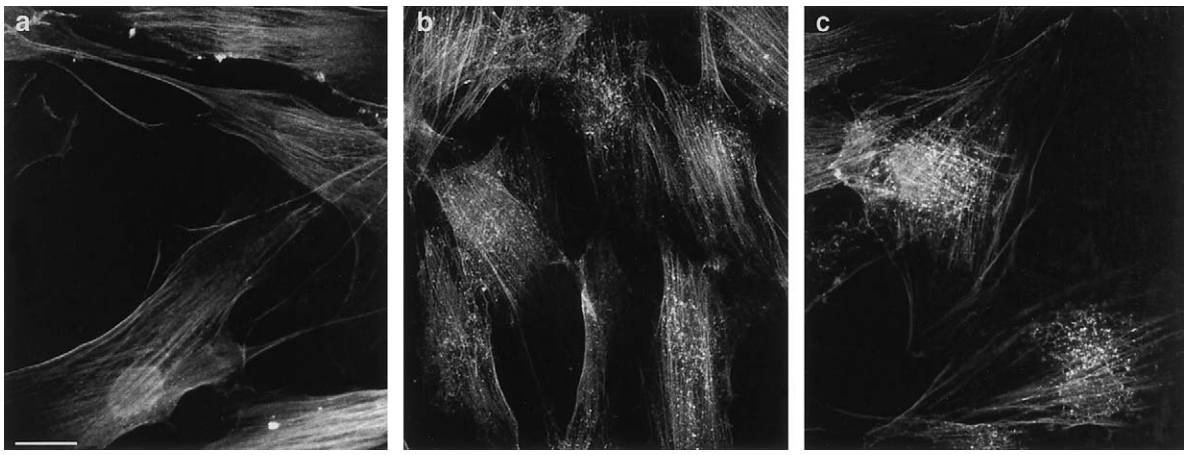

Figure 4 Gelsolin staining is altered in Lowe fibroblasts. Control and two Lowe fibroblast cultures were fixed, permeabilized, and immunostained with a monoclonal antibody to gelsolin, followed by an Alexa 594 conjugated secondary antibody. In control cells (*a*), gelsolin staining was distributed along stress fibers. In Lowe fibroblasts (*b* and *c*), there was an accumulation of punctate staining in the perinuclear area that was virtually absent in normal fibroblasts. Scale bar = 0.02 μm.

tion and permeabilization. The earliest time point at which there was a clear alteration in actin staining in Lowe cells was 5 min after drug treatment; comparisons of staining between Lowe and normal cells are therefore shown at the 5-min time point.

Figure 3 shows untreated control (*a*) and Lowe cells (lines 2 and 4) (*d* and *g*) and control and Lowe fibroblasts treated with cytochalasin D (*b*, *e*, and *h*) or latrunculin A (*c, f,* and *i*). Disruption of the cytoskeleton increased with exposure time in Lowe and control cells, but depolymerization was more rapid in the Lowe cells. Treated Lowe fibroblasts, shown in figure 3 (*e, f, h,* and *i*), had a reduction in F-actin staining in almost all cells, with fewer long actin filaments and an increased number of short filaments. Conversely, normal cells had only a slight change in stress-fiber staining after the short treatment, and many of the cells showed a staining pattern indistinguishable from untreated cells (fig. 3*b* and 3*c*). The more rapid response of Lowe fibroblasts to these agents indicated that Lowe cells had more filament ends and may have an increase in actin-severing activity.

Gelsolin is a major actin-severing protein regulated by PIP_2 and Ca^{+2} (Janmey and Stossel 1987; Janmey 1994; Lin et al. 1997*a*). Elevated levels of PIP_2 in Lowe cells (Zhang et al. 1998) may lead to local alterations in Ca^{+2} levels through the activity of phospholipase C, by which PIP_2 is metabolized to inositol trisphosphate and diacylglycerol, leading to the release of calcium from intracellular stores. Consequently, we investigated whether Lowe cells had a change in the distribution of gelsolin by immunofluorescence (fig. 4), performed essentially as for the actin staining above, except that the cells were incubated with gelsolin monoclonal antibody (Sigma) in PBS with 1% NGS for 1 h, followed by a 1-h incubation

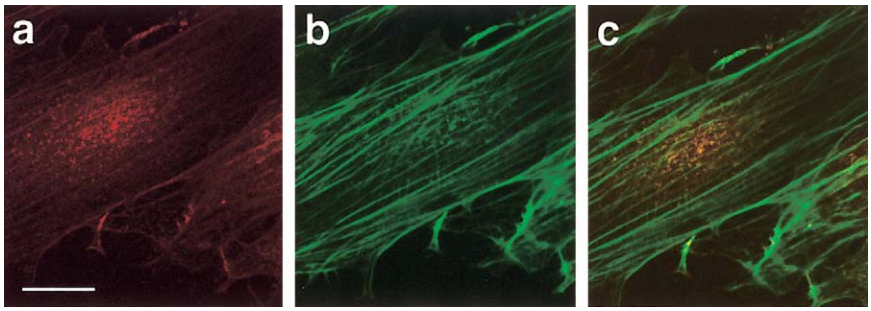

Figure 5 Punctate gelsolin staining in Lowe fibroblasts coincides with the punctate F-actin filament staining. Lowe fibroblasts were fixed and incubated with a monoclonal antibody to gelsolin followed by an Alexa 594 conjugated anti-rabbit secondary antibody, along with Alexa 488 conjugated phalloidin. Fluorescence was visualized by confocal microscopy. The punctate gelsolin staining (*a*) and punctate F-actin staining (*b*) coincided, as shown in the merged image (*c*). Scale bar = 0.02 μm.

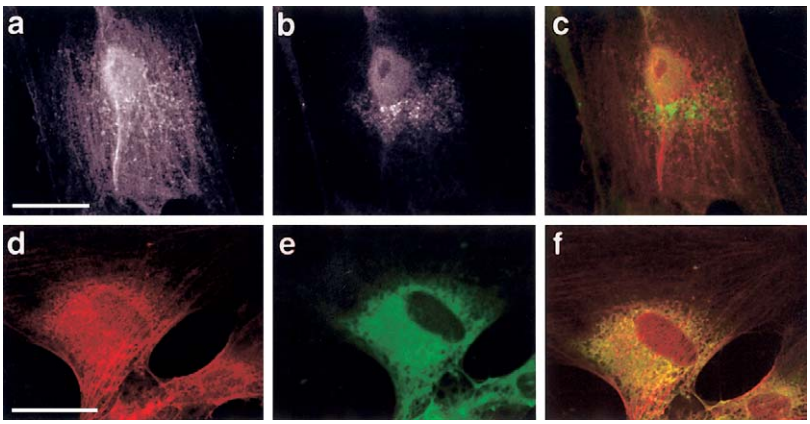

Figure 6 Distribution of the punctate gelsolin staining does not colocalize with the TGN but does overlap with that of an ER marker. Lowe fibroblasts (*a–c*) were fixed and incubated with a monoclonal antibody to gelsolin and a polyclonal antibody to the mannose 6-phosphate receptor (M6-PR), followed by an Alexa 594 conjugated anti-mouse secondary antibody and an Alexa 488 conjugated anti-rabbit secondary antibody. The gelsolin staining (*a*) did not overlap with that of the TGN marker, M6-PR (*b*), as seen in the merged image (*c*). Scale bar = 0.06 μm. The localization of the punctate gelsolin staining was further studied (*d–f*) by double-labeling Lowe fibroblasts, as described above, with anti-gelsolin antibody and with Alexa 488 conjugated concanavalin A, a probe for the endoplasmic reticulum. Fluorescence was visualized by confocal microscopy (*d–f*), scale bar = 0.063 μm. The gelsolin staining (*d*) and the concanavalin A staining (*e*) partially overlapped, as shown in the merged image (*f*).

with an Alexa 594-anti-mouse IgG (Molecular Probes). In control cells, gelsolin staining was present along stress fibers and at the plasma membrane, as reported elsewhere, along with a diffuse nuclear staining (Dissmann and Hinssen 1994) (fig. 4*a*). In Lowe fibroblasts (lines 2 and 5), in addition to the stress-fiber staining, there was a redistribution of gelsolin staining in a punctate pattern in the center of the cell (fig. 4*b,* and 4*c*). This punctate staining was virtually absent from normal fibroblasts. We have confirmed these observations in three additional Lowe fibroblast lines (data not shown). The fact that the same punctate gelsolin staining was observed in the six lines from patients with Lowe syndrome that were tested, including three with missense mutations that abolished PIP_2 5-phosphatase activity but had ocrl1 protein detectable by western analysis (Lin et al. 1997*b,* 1998; Gropman et al. 2000), indicates that the PIP_2 5-phosphatase activity is responsible for the actin abnormality reported here.

Gelsolin not only severs actin filaments but also caps filament ends after severing. To assess whether the punctate staining observed in Lowe fibroblasts represented free gelsolin or gelsolin bound to short actin filaments, we double-labeled Lowe fibroblasts (line 5) using phalloidin and anti-gelsolin antibody and photographed cells with a Zeiss Axiovert 100 confocal microscope. The punctate gelsolin staining (fig. 5*a*) coincided with punctate F-actin staining (fig. 5*b*), in merged images (fig. 5*c*), indicating that, in this region, the short actin filaments were associated with and possibly capped by gelsolin. This points to the possibility that there is a localized increase in gelsolin activity in Lowe fibroblasts.

To determine whether the punctate gelsolin staining in Lowe cells overlapped with the trans-Golgi network (TGN), where ocrl1 is localized in normal cells, we performed double-label immunofluorescence in Lowe cells with gelsolin antibody and a polyclonal antibody to the TGN-localized mannose 6-phosphate receptor (M6-PR), kindly provided by Dr. J. Paul Luzio (Cambridge University) (fig. 6*a–6c*). M6-PR localized to a condensed region in the perinuclear area of the cell (line 5) (fig. 6*b*), whereas the punctate gelsolin staining (fig. 6*a*) appeared in a broader area around the nucleus. In merged images, it was clear that the punctate gelsolin staining was completely excluded from the TGN identified by the M6-PR antibody (fig. 6*c*). Because of the broader cytoplasmic distribution of the punctate gelsolin staining, we double-labeled the cells with an antibody to gelsolin (fig. 6*d*) (line 6) and Alexa 488 concanavalin A as a probe for the endoplasmic reticulum (ER) (fig. 6*e*) (Miralles et al. 1993; Ofner and Hooper 2002). By confocal imaging, we saw a clear overlap of the staining pattern in the merged image (*yellow staining*, fig. 6*f*), demonstrating that the punctate gelsolin staining overlapped the ER.

To confirm that ocrl1 expression was responsible for the punctate gelsolin staining, Lowe fibroblasts that had previously shown to have no detectable OCRL1 mRNA (Olivos-Glander et al. 1995) were transfected with OCRL1 cDNA in a mammalian expression vector. Fi-

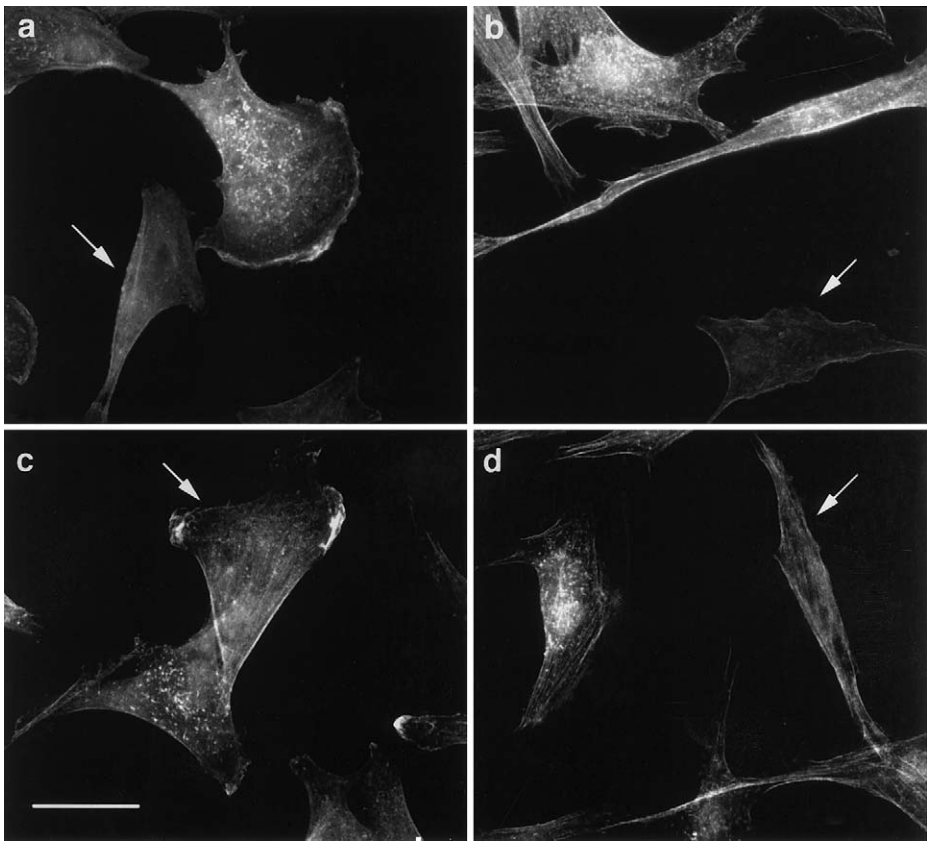

Figure 7 Lowe cells transfected with ocrl1 cDNA are lacking the punctate gelsolin staining seen in most untransfected fibroblasts. Images show gelsolin staining of transfected (*arrows*) and untransfected cells. Following transfection, cells were plated, fixed, and double-labeled with an antibody to ocrl1, to identify transfected cells, and with gelsolin antibody. Scale bar = 0.05 μm.

broblasts from patients with Lowe syndrome (7×10^5 cells) were transfected with 2 μg of OCRL1 cDNA (Olivos-Glander et al. 1995) with the Amaxa Biosystems NHDF-Neo Nucleofector kit using the manufacturer's optimized protocol for human fibroblasts. Cells were plated immediately after transfection onto polylysine-coated glass slides (Nunc), and immunofluorescence was performed after 24–48 h (as described above) with anti-gelsolin monoclonal antibody and ocrl1 polyclonal antibody.

Transfected Lowe fibroblasts were scored for expression of ocrl1. Transfection efficiency in different experiments, with two different Lowe cell lines (line 1 and line 2), was estimated by comparing the percentage of ocrl1 stained cells to total cells in several fields, which ranged from 1% to 12%, although, in most experiments, efficiency was 1%–2%. This efficiency is significantly lower than that reported by the manufacturer. According to the manufacturer, their transfection efficiency experiments were done with first passage newborn dermal fibroblasts and might be diminished considerably by the age of the donor, the passage number of the cell line, the length of time the cell line had been frozen, and the plasmid used in transfections. Although the punctate staining was present in the majority of untransfected fibroblasts (fig. 7*a*–7*d*), as described above, no transfected cells (fig. 7, *arrows*) had punctate staining (fig. 7*a*–7*d*). In the experiment shown here, both transfected and untransfected cells appeared to have reduced stress-fiber staining.

Additional actin-binding proteins were studied in normal and Lowe fibroblasts: profilin, cofilin, vinculin, villin, and α-actinin. Of these, only α-actinin was found to have an abnormal staining pattern in Lowe fibroblasts. For α-actinin immunofluorescence, cells were fixed for 5 min with cold (-20°C) methanol, washed, blocked, and incubated with primary and secondary antibodies (as described above) using α-actinin monoclonal antibody (Sigma), followed by Alexa 488–anti-mouse IgM. Cells of both genotypes displayed an even distribution of the α-actinin protein along actin stress fibers and concentrated at focal adhesions and at the plasma

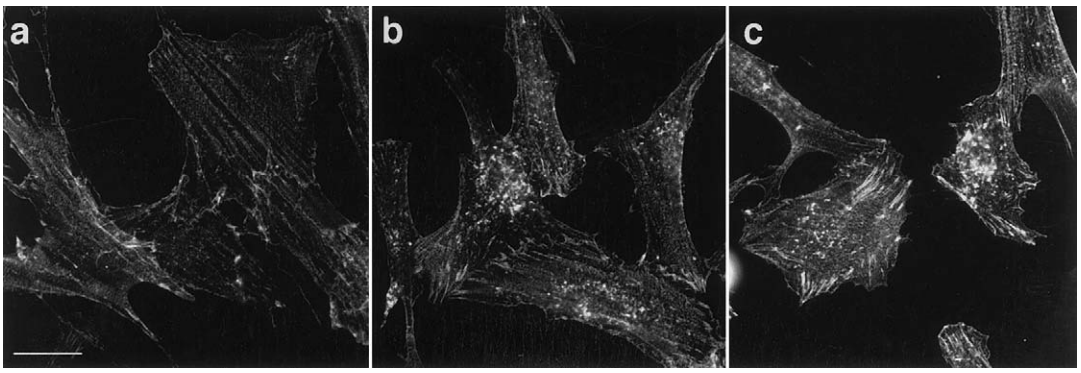

Figure 8 Immunofluorescence staining of α-actinin is altered in Lowe fibroblasts. Control and two Lowe fibroblast cultures were fixed, permeabilized, and immunostained with a monoclonal antibody to α-actinin followed by an Alexa 488 conjugated secondary antibody. In control cells (*a*), α-actinin staining was present at focal adhesions and along stress fibers. In Lowe fibroblasts (*b* and *c*), there was an accumulation of punctate staining in the perinuclear area that was virtually absent in normal fibroblasts. Scale bar = 0.05 μm.

membrane (fig. 8*a*–8*c*), as reported elsewhere (Edlund et al. 2001). However, in addition to this staining, Lowe fibroblasts showed a distinctly abnormal accumulation of punctate staining in the center of the cell in ~61% of Lowe fibroblasts (fig. 8*b* and 8*c*) (lines 2 and 1), whereas only 4% of normal fibroblasts had similar staining. It is interesting that α-actinin activity, like that of gelsolin, is dependent upon PIP_2 and Ca^{+2}. Double-label immunofluorescence studies to determine whether the punctate α-actinin and gelsolin staining overlapped were unsuccessful, since the α-actinin and the gelsolin antibodies were both mouse monoclonals, and the optimum fixation conditions for the detection of α-actinin staining were not compatible with detection of gelsolin staining.

We found alterations in the actin cytoskeleton in Lowe fibroblasts: a reduction in number and length of actin stress fibers and an altered distribution of two actin-binding proteins, gelsolin, and α-actinin. These abnormalities are a consequence of elevated PIP_2 levels (and possibly of local increases in Ca^{+2}) that may be present in particular subcellular domains. This novel cellular phenotype is likely to be a contributory cause of the clinical phenotype in Lowe syndrome. Further study will be necessary to determine the precise relationship between the cellular and clinical phenotypes and why only certain tissues are affected. Certain cell types or subcellular structures may be more susceptible to chronic abnormalities in actin or actin-binding proteins. Two of the major defects in Lowe syndrome appear to be in renal and lens epithelium, leading to renal tubular dysfunction and a failure of the primary lens fiber cell differentiation (Nussbaum and Suchy 2001). It has been shown that epithelial cell adhesion requires interaction of actin with actin-binding proteins such as E-cadherin and α-catenin (Adams et al. 1998; Vasioukhin et al. 2000), and that these cell-cell contacts fail to form when actin polymerization is disrupted (Vasioukhin et al. 2000). Actin polymerization plays a key role in the formation, maintenance, and proper functioning of tight junctions in cultured kidney epithelium (Stevenson and Begg 1994; Wittchen et al. 1999) that are required for normal barrier function of the renal proximal tubule cells (Cereijido et al. 1993). Remodeling of the cytoskeleton has also been demonstrated to be essential for lens differentiation (Lee et al. 2000), and the lens fails to develop properly when F-actin and cell-adhesion molecules fail to organize at membranes (Ferreira-Cornwell et al. 2000).

We hypothesize that Lowe syndrome may result from a chronic deficiency of a PIP_2 5-phosphatase, localized to the TGN (Dressman et al. 2000), with a consequent elevation of PIP_2 that can effect the actin cytoskeleton and, in turn, the formation and maintenance of certain cell-cell contacts. The identification of this cellular phenotype provides a model system to test possible interventions in the disease process and perhaps to interrupt pathogenesis in this disorder.

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Electronic-Database Information

Accession numbers and URLs for data presented herein are as follows:

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for OCRL [MIM 309000])

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