High levels of epidermal growth factor (EGF)-receptors have been reported in membrane homogenates of bovine retinas, but the biologic function and tissue target of EGF in the retina have not been established fully. Because EGF participation has been suggested in the mechanisms of wound healing and Müller cells undergo changes after retinal injury, the authors studied EGF receptor expression and functional role of this substance in cultured Müller cells. These cells (isolated from normal rats) were tested for the glial cell markers: vimentin, S-100 protein, and carbonic anhydrase C. These markers were found to be positive through all passages used in the experiments. The $^{125}$I-EGF binding in Müller cells was highly specific, concentration dependent, and saturable. Compared with 3T3 fibroblasts, Müller cells bound threefold more EGF. Binding kinetics and Scatchard analyses showed the higher level of binding was related to the greater number of receptors on these cells (Müller cells, $2.4 \times 10^5$ receptors/cell; 3T3 fibroblasts, $7.1 \times 10^4$ receptors/cell) rather than a change in affinity of the receptors to bind the ligand. Nonlinear-regression analyses suggested the presence of two classes of affinity sites. The high level of EGF-receptor expression in Müller cells was confirmed by western blot analyses that showed increased reactivity of the approximately 170-kilodalton receptor band to a monoclonal anti-EGF receptor antibody. Moreover, EGF treatment of Müller cells resulted in two- to threefold increase in DNA synthesis, as evidenced by $^3$H-thymidine uptake studies. These findings support a functional role for EGF in Müller cell proliferation in retinal disease.

After brain trauma or disease, glial cells undergo a series of morphologic and biochemical changes, including migration, proliferation, and increased glial fibrillary acidic protein (GFAP) expression in astrocytes. Although the mechanisms in the glial cell response to injury or disease are largely unknown, growth factors involved in wound repair have been linked to glial cell changes during culture. Fibroblast growth factors stimulate proliferation and increase GFAP expression. Platelet-derived growth factor is chemotactic to brain astrocytes. Epidermal growth factor (EGF) is mitogenic and stimulates GFAP expression, c-fos induction, and protein phosphorylation in brain-derived glial cells. It also stimulates cell growth and migration in human tumor cell lines of glial origin. These studies support a role for growth factors, especially EGF, in glial cell changes during brain injury or disease.

In the retina, Müller cells undergo changes similar to those observed in astrocytes after brain injury. Reports of high levels of EGF messenger RNA and receptors in bovine retinal extracts convinced us that EGF also might be important in modulating Müller cell changes, including those observed after retinal injury. Therefore, we investigated the role of EGF in Müller cell changes during retinal injury or disease. Preliminary studies were done to characterize EGF-receptor expression in cultured Müller cells and to test EGF effects on Müller cell proliferation.

**Materials and Methods**

**Preparation and Characterization of Müller Cells**

We used RCS-rdy rats (congenic control animals for Royal College of Surgeons rats) that were 8–10 days old. They were killed in accordance with the ARVO Resolution on the Use of Animals in Research. Their eyeballs were removed, and Müller cells were isolated using a technique described previously. Briefly, the eyeballs were soaked in Dulbecco’s modified Eagle’s medium (DMEM) containing 2 mmol/L glutamine, 100 units/ml of penicillin, and 100 µg/ml of streptomycin for 12–16 hr. Then
they were incubated in DMEM containing 1 mg/ml of trypsin, 0.4 mg/ml of ethylenediaminetetraacetic acid, and 70 units/ml of collagenase at 37°C for 1 hr. The retinas were isolated, separated manually by repeated pipetting, and cultured in DMEM supplemented with 10% fetal bovine serum (DMEM/FBS10). The cells were allowed to grow in culture for 5–7 days; after this, the medium was changed. They were grown to confluence and passaged every 7 days. All cultures, unless specified otherwise, were grown in DMEM/FBS10 in a humidified atmosphere of 95% air and 5% CO2 at 37°C. All chemicals, culture media, and growth supplements were obtained from Sigma (St. Louis, MO).

The Müller cells we used were characterized during each passage. The cells were grown on glass cover slips for 48 hr and fixed with 2% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.3) for 20 min. They were incubated in 2% normal goat serum (Vector, Burlingame, CA) for 1 hr. Then monoclonal antivimentin immunoglobulin G (1:10; Lipshaw, Detroit, MI), polyclonal antianalytic anhydrase C immunoglobulin G (1:500; a gift from Dr. Debra Hazen-Martin, Medical University of South Carolina, Charleston, SC), or polyclonal antiovine brain S-100 protein immunoglobulin G (1:200; DAKO, Carpinteria, CA) was added at 4°C overnight. After rinsing, the cells were incubated for 1 hr in fluorescein-labeled goat anti-mouse or goat anti-rabbit immunoglobulin G (Sigma), depending on the primary antibody used. All rinses were done using PBS with 1% bovine serum albumin (BSA). For control experiments, duplicate cultures were processed in the absence of the primary antibody. To test for contamination with other cell types, the cells were also processed for specific cell markers using the following monoclonal antibodies: anticytokeratin pan immunoglobulin G1 (1:10; Boehringer Mannheim, Indianapolis, IN) for retinal pigment epithelial cells, antineurofilament 200-kilodalton immunoglobulin G1 (1:200; Boehringer Mannheim) for neurons, and anti-GFAP immunoglobulin G (1:6; Lipshaw) for astrocytes. Polyclonal antifactor VHI-related antigen immunoglobulin G (1:50; DAKO) was used to identify endothelial cells. Produced using an electronic Coulter (Hialeah, FL) counter.

The data were analyzed statistically using nonlinear-regression techniques with a least-squares curve-fitting program16 based on the Marquardt-Levenberg algorithm. The kinetics of 125I-EGF binding to Müller cells were analyzed based on ligand binding to a single population of noninteracting sites or to two binding sites. The appropriateness of each model was evaluated using an F test.

EGF-Receptor Immunolabeling

Müller cells and 3T3 cells, after culturing on glass cover slips in DMEM/FBS10 for 48 hr, were processed for immunocytochemical analysis as mentioned previously (see Müller cell characterization) except we used a monoclonal anti-EGF receptor immunoglobulin G1 (1:500; Sigma). Produced using paraformaldehyde-fixed A431 cells as the immunogen, this antibody recognizes a carbohydrate moiety in the EGF-receptor extracellular domain. To compare the relative amounts of reactivity to the EGF-receptor antibody, confluent cultures of Müller cells and 3T3 fibroblasts in T75 flasks were processed for sodium dodecyl sulfate–polyacrylamide gel electrophoresis17 and immunoblotting. The cell monolayers were placed on ice, rinsed twice, and harvested in ice-cold PBS containing the protease inhibitor, phenylmethanesulfonyl fluoride (PMSF). They were centrifuged at 80 X g at 4°C for 10 min. The
pellet was dissolved in lysis buffer consisting of 20 mmol/1 Tris, 137 mmol/1 NaCl, 1 mmol/1 PMSF, and 1% Triton-X-100 for 30 min. The lysates were centrifuged at 15,000 × g at 4°C for 10 min, and the supernatants were stored either at −70°C or used immediately. Aliquots of 20 μg of solubilized samples were loaded onto 10% acrylamide gels. Proteins and pre-stained molecular weight standards (26,600–186,000 Daltons) were transferred to nitrocellulose membranes for immunoblotting. A431 membrane vesicles, isolated using Thom’s technique, served as the positive control for the EGF receptor. Human epidermoid carcinoma A431 cells have been used widely in EGF binding studies because of their numerous receptors.9

Nitrocellulose membranes were blocked with 2% normal horse serum in PBS/BSA for 1 hr and incubated with the anti-EGF receptor antibody at a 1:1000 dilution overnight at 4°C. This was followed by incubation in biotinylated horse anti-secondary antibody diluted 1:200 in PBS/BSA. Then horseradish peroxidase-conjugated avidin-biotin complex (Vectastain ABC kit; Vector) was added. The reaction was completed by adding 0.015% H2O2 and 0.05% 4-chloro-1-naphthol in PBS. All immunochromat-ics were obtained from Vector. Peak areas of EGF-receptor reactive bands in the blots were measured using a Shimadzu CS-9000 scanning densitometer (Kyoto, Japan).

DNA Synthesis

Müller cells (8th–15th passages) and 3T3 fibroblasts grown to confluence in 22-mm wells were incubated in DMEM supplemented with various serum concentrations (0.25%, 1.0%, or 10.0% FBS) for 20 hr. The EGF was added at various concentrations (1–100 ng/ml), and the cultures incubated for another 18 hr. After this time, 1 μCi of 3H-thymidine (71 Ci/mmol; ICN) was added, and the incubation was continued for another 3 hr. The cell monolayers were placed on ice, rinsed three times with ice-cold EBSS/BSA, covered with 0.5 ml of 5% trichloroacetic acid (TCA) overnight, and rinsed three times with 5% TCA to remove unincorporated 3H-thymidine. The TCA-insoluble material was dissolved in 0.5 ml of 1 N NaOH. The samples were transferred to vials and neutralized with 6N HCl. Radioactivity was measured in a Beckman LS 1801 scintillation counter using Scintiverse II fluid (Fisher, Norcross, GA). All experiments were done in triplicate.

Results

Characteristics of Cultured Müller Cells

Müller cells were prepared from normal rats using an earlier technique.15 This requires a soaking treat-ment that stimulates cell division and outgrowth of Müller cells from the degenerating retina. Using this procedure, Müller cells isolated during early passages were found to be mononucleated spindle-shaped cells with thin branching processes (Fig. 1A). After several passages, cell cultures maintained at low density in 10% serum-containing medium resulted in flattening of the cells, hypertrophy, and loss of branching processes (Fig. 1E). Early-passaged Müller cells were positive for glial cell markers: vimentin, S-100 protein, and carbonic anhydrase C (Figs. 1B–1D). Although vimentin is a nonspecific glial cell marker, carbonic anhydrase C and S-100 protein are considered reliable markers for Müller cells.20,21 The cultures were negative for the retinal pigment epithelial cell marker (cytokeratin), the neuronal marker (neurofilament 200 kilodalton), and the endothelial cell marker (Factor VIII-related antigen) at all passages used in our experiments (data not shown). A few contaminating cells were positive for G. simplicifolia isoelectin B4 (Fig. 1E), which is specific for α-galactose residues and has been reported to be a useful marker for endothelial cells22 and microglia.23 F-actin filaments labeled with rhodamine phalloidin also were observed in Müller cells but were absent in cells labeled with G. simplicifolia. Occasional cells also contained GFAP, which is specific for astrocytes (Fig. 1F). G. simplicifolia- and GFAP-reactive cells comprised approximately 5–10% of the total cell population.

Increased EGF Receptor Expression in Müller Cells

We found increased EGF binding in Müller cells with increasing concentrations of the radiolabeled ligand; this was saturated at approximately 80 ng/ml (Fig. 2A). Saturation was observed earlier in 3T3 cells at EGF concentrations of approximately 40 ng/ml. At saturating concentrations, Müller cells bound three- to fourfold more EGF than 3T3 cells. Nonspecific binding included no more than 10% of total radioactivity bound to the cells. Using Scatchard analysis (Fig. 2B), we calculated EGF receptor numbers and affinity and found approximately 2.4 × 105 receptors/cell with a dissociation constant (Kd) of 2.2 × 10−9 mol/l in Müller cells and 7.1 × 104 receptors/cell with a Kd of 2.8 × 10−9 mol/l in 3T3 fibroblasts. Although EGF binding in both cell types resulted in a curvilinear plot in the Scatchard analyses, linear curves were fitted using single-binding site models for purposes of simplification. This also allowed us to compare EGF binding in our control cells with binding kinetics in 3T3 cells established previously by Scatchard analysis.24,25 Our data showed the receptor number and affinity for EGF in Swiss 3T3 fibroblasts were similar to those previously studied (approximately × 104 receptors/cell with a Kd of 2.7 × 10−9 mol/l).
Fig. 1. Early passaged Müller cells were spindle-shaped with long branching processes (A). Müller cells were immunoreactive for vimentin (B). Punctate staining was observed with S-100 (C) and carbonic anhydrase-C (D). The most intense reaction in S-100 was localized in the nuclear area, whereas labeling with carbonic anhydrase-C was primarily perinuclear. Müller cells grown at low density in high serum became flattened and hypertrophic and they lost their processes (E). A few contaminating cells were reactive for Griffonia simplicifolia (GS), isolectin B4 (E), or glial fibrillary acidic protein (F). F-actin filaments observed in the Müller cells were absent in GS isolectin B4 reactive cells (arrows). (A, X60; B-F, X122.)

Nonlinear-regression analysis of EGF binding in Müller cells was done using curve-fitting procedures based on single- or double-binding site models (Fig. 3). The receptor numbers and $K_d$s were measured for each model. For the single-binding site model, Müller cell EGF receptors were estimated to be approximately $2.9 \times 10^5$ per cell with a $K_d$ of $2.2 \times 10^{-9}$ mol/l. These values were comparable to values derived using Scatchard analysis. For double-binding sites, high-affinity receptors were computed at approximately 3.5
Fig. 2. EGF binding (A) to cultured Müller cells (filled circles) saturated at epidermal growth factor (EGF) concentrations of \(~80\) ng/ml. Binding in 3T3 cells (open circles) saturated at \(~40\) ng/ml. At saturating concentrations, Müller cells bound \(3-4\) times more EGF than 3T3 cells. For purposes of simplification, Scatchard analyses (B) of EGF binding in Müller cells (filled squares) and 3T3 cells (open squares) were done based on a single binding site model. The almost parallel orientation of the linear plots indicated the increased binding in Müller cells was the result of a higher number of receptors, rather than a change in the affinity of the receptors to bind the ligand.

\(10^4\) per cell with \(K_d\) of \(1.5 \times 10^{-10}\) mol/l; low-affinity receptors were approximately \(2.8 \times 10^5\) per cell with \(K_d\) of \(3.6 \times 10^{-9}\) mol/l. The estimated number of high-affinity receptors was 10% of the total receptor number; this was consistent with previous findings of 5–10% high-affinity EGF receptors in some cell types studied.\(^{25}\) An F test showed that the double-binding-sites model provided a significantly better fit for the binding data compared with the single-binding-site model \((P < 0.05)\).

Labeling with anti-EGF-receptor immunoglobulin G was observed as punctate staining in both Müller cells and 3T3 fibroblasts (Fig. 4). To compare the relative amounts of EGF-receptor protein expression in Müller cells and 3T3 cells, confluent cultures of both cell types were processed for gel electrophoresis and western blotting. Immunoreactive bands were observed in both cell types at approximately 170 kilodaltons (Fig. 5), at the same position as that seen by A431 membrane preparation. A higher mobility band, observed in Müller cells at approximately 110 kilodaltons (or 150 kilodaltons in other instances, data not shown) was just visible in 3T3 cells, but it was intense in the A431 membrane proteins used as control. Densitometric scanning of the EGF-receptor immunoreactive 170-kilodalton bands showed peak areas of approximately \(1.2 \times 10^3\) in 3T3 cells and \(2.2 \times 10^3\) in Müller cells, an approximately 1.78-fold increase in Müller cells. Densitometric scanning of the additional band at 110 kilodaltons in Müller cells showed a peak area of \(2.4 \times 10^3\). When the peak areas of the 170- and 110-kilodalton bands in Müller cells were combined, the total area of \(4.6 \times 10^3\) was approximately 3.8-fold higher than the lone immunoreactive band in the 3T3 cells. This finding was consistent with the three- to fourfold increase in EGF-receptor binding observed in Müller cells.

Increased DNA Synthesis in EGF-Treated Müller Cells

To determine the mitogenic effects of EGF, Müller cells grown in various serum concentrations were
Fig. 4. Intense epidermal growth factor receptor immunoreactivity was observed in the form of punctate staining in Müller cells (A) and 3T3 cells (B). (x120.)

treated with different concentrations of EGF (1–100 ng/ml). Under all conditions studied, DNA synthesis, as measured by $^3$H-thymidine incorporation into TCA-insoluble material, was increased after EGF treatment (Fig. 6). Müller cells grown in 0.25%, 1%, or 10% serum responded with increased $^3$H-thymidine uptake after EGF treatment. We found EGF concentrations from 1–100 ng/ml elicited comparable mitogenic effects on Müller cell cultures. The increase in $^3$H-thymidine uptake in Müller cells after EGF treatment also was compared with that in 3T3 cells treated with EGF. The $^3$H-thymidine labeling in 3T3 cells cultured in 1% serum after treatment with 1–100 ng/ml EGF was 1.3–1.5-fold higher compared with control cultures (Table 1). Under the same experimental conditions, labeling in Müller cells resulted in a 2.4–2.8-fold increase, reflecting the high sensitivity of Müller cells to EGF.

Discussion

Müller cells are the predominant type of glial cells in the retina. Their widespread distribution and intimate relationships with neurons and microvascular endothelial cells allow them to perform important functions in the retina, including nutrition, carbon dioxide and pH regulation, potassium-ion spatial buffering, and synthesis and renewal of visual pigments. During retinal injury or disease, Müller cells undergo morphologic alterations and hypertrophy, show abnormal accumulation of GFAP, proliferate, and participate in chemotaxis. For example, displaced Müller cells have been identified in diabetic preretinal membranes. In several rodent models of retinal dystrophy, increased expression of GFAP was found in Müller cells, and migration and proliferation of their processes in the subretinal space have been observed. We decided to investigate the biochemical and molecular basis for Müller cell alterations, specifically, the role of growth factors, during retinal injury or disease. Our preliminary studies

Fig. 5. Western blot shows immunolabeling of ~170 kD bands in Müller cells (MC), 3T3 cells, and A431 membrane vesicles. Densitometric scanning (bottom) of immunoreactivity of ~170 kD band showed a peak area of $2.2 \times 10^5$ in Müller cells and $1.2 \times 10^5$ in 3T3 cells, a 1.78X increase. An ~110 kD band in Müller cells was hardly visible in 3T3 cells.
measured EGF-receptor expression and its effects on DNA synthesis in cultured Müller cells.

We studied EGF-receptor expression in Müller cells using a radiolabeled ligand-binding assay. We found that EGF binding in Müller cells was highly specific, concentration dependent, and saturable. Moreover, EGF binding in Müller cells was three- to fourfold higher than the amount seen in 3T3 cells. Scatchard analyses showed that this increased level of EGF binding in Müller cells was a result of a three- to fourfold greater number of receptors in Müller cells compared with 3T3 cells rather than a change in the affinity of the receptors to bind EGF.

The total specific binding of EGF in cultured cells included receptor-bound EGF and internalized receptor-EGF complexes. Because internalization of the receptor-bound ligand increased with temperature, our studies (done at room temperature) may reflect a large amount of internalized receptor-EGF complexes, resulting in increased levels of binding. However, the close similarity in the number of EGF receptors and binding affinity in our control 3T3 cultures and in 3T3 fibroblasts studied earlier under the same experimental conditions24,25 support our conclusion that Müller cells have more EGF receptors.

Scatchard plots of EGF binding in Müller cells and 3T3 cells in our studies generated curvilinear plots, indicating the presence of two binding sites. Therefore, the binding kinetics in Müller cells were evaluated further using nonlinear-regression analyses. Scatchard analysis of radioligand-binding data requires transformation of these data into linear relationships; this change could cause less optimum results, especially when dealing with nonlinear relationships.35 Curves based on single- or double-binding sites were fitted to the binding data and showed a significantly better fit for the two-binding-site model, supporting the presence of two affinity binding sites in Müller cells. Moreover, the high-affinity receptors were calculated to be approximately 10% of the total receptor number; this result was similar to a previous report of 5–10% high-affinity EGF receptors in certain cell types.19

The presence of more than one type of EGF receptors in Müller cells was consistent with previous reports of high- and low-affinity sites in human epidermoid carcinoma cells, keratinocytes, and HeLa cells (as determined by equilibrium binding experiments, Scatchard plot analyses, or competitive binding using monoclonal antibodies against EGF receptors).19,36–38 The high-affinity sites mediate early cellular responses to EGF, such as phosphorylation of the EGF receptor, inositol phosphate turnover, release of calcium ion from intracellular stores, induction of c-fos gene expression, and increased intracellular pH. Thus, our findings may serve as important baseline data for future studies of EGF modulation of Müller cell changes.

The increased level of EGF-receptor expression in Müller cells was confirmed by immunoblot studies showing increased labeling of the approximately 170-kilodalton EGF-receptor band in Müller cells compared with 3T3 cells. Although Müller cells are bigger than 3T3 cells (which could result in higher numbers of receptors per cell), western blots that were equal-

Table 1. Effects of epidermal growth factor (EGF) on 3H-thymidine labeling in Müller cells and 3T3 fibroblasts cultured in 1% serum

<table>
<thead>
<tr>
<th>EGF concentration (ng/ml)</th>
<th>Müller cells</th>
<th>3T3 cells</th>
<th>Ratio of increase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cpm</td>
<td>cpm</td>
<td>Müller cells</td>
</tr>
<tr>
<td>0</td>
<td>66,280 ± 3,800</td>
<td>359,213 ± 15,026</td>
<td>—</td>
</tr>
<tr>
<td>1</td>
<td>187,980 ± 4,130</td>
<td>476,273 ± 14,740</td>
<td>2.8X</td>
</tr>
<tr>
<td>10</td>
<td>183,620 ± 1,113</td>
<td>555,393 ± 24,235</td>
<td>2.8X</td>
</tr>
<tr>
<td>100</td>
<td>157,347 ± 4,324</td>
<td>527,173 ± 13,678</td>
<td>2.4X</td>
</tr>
</tbody>
</table>

cpm, counts per minute.
ized for total cellular protein showed a substantial increase in the EGF receptor protein in Müller cells compared with 3T3 cells. This suggests that the receptor density and number was greater in the Müller cells than the 3T3 fibroblasts.

DNA synthesis (evaluated by the rate of \(^{3}H\)-thymidine incorporation into TCA-insoluble material) was increased 2.4-2.8-fold in EGF-treated Müller cells compared with control cultures, reflecting the higher sensitivity of Müller cells to this growth factor. Moreover, EGF at various concentrations showed almost similar mitogenic effects. However, at high concentrations, its effects on some transformed cell lines, such as A431 cells, were inhibitory. This suggests that low EGF concentrations in the retinal milieu may be enough to elicit the maximum mitogenic response. Furthermore, the continued presence of high EGF concentrations during retinal injury may provide continuous stimulation of Müller cell proliferation.

The mechanisms involved in the control of the glial cell response to injury or disease are largely unknown. Most studies done on the brain and on brain-derived tissues suggest that growth factors, such as EGF,\(^{2-11}\) may be important in glial cell changes during disease. Recent reports of high levels of EGF message RNA and receptors in bovine retinal extracts\(^{14}\) convinced us that EGF also might be important in the retina. Our study shows that EGF binding in cultured Müller cells was high, and this high level of binding was not a result of an increase in affinity for EGF but was related to a higher number of EGF receptors. Moreover, EGF treatment caused increased DNA synthesis in cultured Müller cells. Our study, therefore, identified the Müller cell as a major target for EGF action in the retina. In addition, the greater number of EGF receptors in Müller cells compared with most cell types studied (including brain-derived cells, 4-10 \times 10^3 per cell in astrocytes, 6.6-10 \times 10^3 per cell in oligodendrocytes, and 1.5-1.9 \times 10^3 per cell in neurons\(^{9}\)) suggested that Müller cells are highly sensitive to EGF.

During retinal injury, EGF release, whether a result of increased (1) secretion by retinal cells or blood-borne migratory cells or (2) extravasation after breakdown of the blood-retinal barrier, may lead to Müller cell proliferation. Moreover, the increased levels of EGF in the retina during disease may result in other stimulating effects, such as increased expression of c-fos and protein phosphorylation, leading to Müller cell transformation. Preliminary studies in our laboratory indicate increased tyrosine phosphorylation of the EGF receptor and other Müller cell proteins after EGF treatment (unpublished data).

In conclusion, Müller cell alterations (believed to play a primary role in inducing subsequent cellular events during retinal disease)\(^{22,29}\) may be caused by the increased stimulating effects of EGF in diseased retinas. Although our study does not prove that such conditions occur in the diseased retina, the increased EGF receptor expression and the mitogenic effect of EGF on the Müller cell support this idea. Additional investigation will be necessary to define the exact role of EGF in modulating Müller cell alterations in retinal disease.

**Key words:** epidermal growth factor receptor, cultured Müller cells, DNA synthesis, carbonic anhydrase C, S-100 protein

**Acknowledgments**

The authors thank Dr. Robert S. Aronstam for his help in the nonlinear-regression analyses; Dr. Stephen R. Ikeda, who allowed the authors the use of his Nonlinear program; Dr. Debra Hazen-Martin, who provided the anticarbonic-anhydrase C antibody; and Myles Black, Jean Doris McCool, Janne T. Jones, and Li Yan Ye for their excellent technical assistance.

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