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Pulmonary preconditioning, injury, and inflammation modulate expression of the candidate tumor suppressor gene *ECRG4* in lung

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Abstract

Purpose—The human c2orf40 gene encodes a candidate tumor suppressor called Esophageal Cancer-Related Gene-4 (*ECRG4*) that is a cytokine-like epigenetically-regulated protein that is characteristically downregulated in cancer, injury, inflammation, and infection. Here, we asked whether *ECRG4* gene expression is detectable in lung epithelial cells and if its expression changes with inflammation, infection, and/or protective preconditioning.

Materials and Methods—We used immunoblotting, PCR, and quantitative PCR to measure ECRG4 and either inhalation anesthesia preconditioning, lipopolysaccharide injection, or laparotomy to modulate lung inflammation.

Results—Immunoblotting establishes the presence of the full-length 14 kDa ECRG4 peptide in mouse lung. Immunohistochemistry localizes ECRG4 to type l alveolar epithelial cells. Basal ECRG4 mRNA is greater than TNF-α, IL-1β, and IL-6 but following inflammatory lung injury, TNF-α, IL-1β, IL-6, and IL-10 are upregulated while *ECRG4* gene expression is decreased. Similar findings are observed after an intravenous administration of lipopolysaccharide. In contrast, lung preconditioning with isoflurane anesthesia increases lung *ECRG4* gene expression. Over-expression of ECRG4 in human lung epithelial cells in vitro decreases cell proliferation implying that a loss of ECRG4 in vivo would be permissive to cell growth.

Conclusions—This study supports the hypothesis that ECRG4 acts as a sentinel growth inhibitor in lung alveolar epithelial cells. Its downregulation by injury, infection, and inflammation and upregulation by preconditioning supports a role for ECRG4 in regulating the alveolar epithelium response to injury and inflammation. By extension, the findings support a functional

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Some of these studies were presented by Ashkaun Shaterian at the Eighth Annual Academic Surgical Congress February 7th, 2013 and published in abstract form [67].

Conception and design of experiments AB, ADM, AS, BPE RC and SK; Analyses and interpretation of the data AB, ADM, BPE, AS, SK, TC, and RC; Drafting manuscript for important intellectual content: AB, ADM, AS, BPE, SK, TC, and RC; Immunoblotting HBC and XD; PCR and qPCR: DMC and XD; immunohistochemistry: SK and Surgery AS.

consequence to its inhibition by promoter hypermethylation (i.e. lung cancer) and suggest potential benefits to its upregulation.

Keywords

c20rf40; epithelial to mesenchymal transition; infection; inflammation; precondtitioning; tumor suppressor gene

INTRODUCTION

The inflammatory sequelae from pulmonary injury can range from edema and acute respiratory distress syndrome (ARDS) to chronic fibrosis and restrictive lung disease. It is therefore critical to understand the molecular mechanisms that regulate the epithelial cell response to inflammation and the factors that might gauge the scope of epithelial responsiveness to drive injury resolution [1–4].

The host pulmonary response to injury is a dynamic process that involves the up- and downregulation of factors that are differentially regulated at both genetic and molecular levels [5, 6]. Because the steady-state proliferative index of the airway epithelial cells is normally very low, epithelial cell plasticity represents a critical element of the pulmonary response to inflammation. For example, as little as 1% of bronchial epithelial cells enter the cell cycle over 24 hours [2]. Yet, in a process that is analogous to the changes that accompany wound healing in other tissues [1, 4, 7], the acute inflammatory–injury response in lung results in a rapid increase in cell de-differentiation, an increase in progenitor cell migration, proliferation, and ultimately, resolution of inflammation through epithelial re-differentiation to quiescence. Accordingly, lung alveolar epithelial responsiveness, recovery and restitution are dynamic processes that involve initiating and terminating factors that regulate the innate capacity of epithelial cells to de-differentiate, migrate, and proliferate [3, 8]. This dynamic capacity for epithelial-to-mesenchymal transition (EMT) is regulated by epidermal, fibroblast, and transforming growth factors, chemokines, interleukins, β-catenin, Rho GTPases, and mechanical forces that have been shown to play key roles in lung epithelial repair [1, 9, 10].

As part of the myriad processes that participate in epithelial cell regulation, constitutive basal gene expression maintains homeostatic set points that precondition the host's capacity to gauge the response to injury and ultimately, to define and drive recovery [11, 12]. Accordingly, "sentinel genes" encode a subset of ligands and receptors that regulate the capacity of the host to respond to inflammation [13–16] and, as such alter the natural course of recovery [17–19]. In this capacity, these sentinel mechanisms (i.e. protective preconditioning) modulate the spectrum injury responses, influence recovery [20–22], and ultimately, alter clinical outcomes [18]. Genes that play sentinel functions have been described in several organs, however, few studies have evaluated the alveolar epithelium for sentinel gene candidates capable of gauging the host response to injury [23, 24]. The human *c2orf40* gene and its product, Esophageal Cancer-Related Gene-4 (*ECRG4*), a candidate tumor suppressor protein [25–31] is a case in point (reviewed in [32]). ECRG4 was originally discovered because it is epigenetically downregulated in various human epithelial

malignancies [31, 33–36] and its promoter is a prototypic target for epigenetic DNA methylation in cancer [25, 28, 31, 37–42]. Furthermore, ECRG4 is a small molecular weight and biologically active protein that is highly conserved across mammalian species [27, 32, 43]. Like many cytokines and growth factors, it is also expressed in diverse mammalian tissues and has multiple activities that depend on its location and post-translational processing [27–29, 36, 40, 44–49].

Several recent studies suggest that ECRG4 plays a role in injury homeostasis because its downregulation after injury/inflammation allows for dysinhibition of cell proliferation in vivo [27, 29, 46, 50, 51]. Its epigenetic regulation, however, suggests that the return of basal gene expression after injury could depend on epigenetic factors determined by environment, diet, and age. These are the same factors that influence outcomes from pulmonary injury and infection. Accordingly, we hypothesized that *ECRG4* gene expression in lung could be affected by preconditioning and in turn, influence the host injury response to pulmonary injury.

MATERIALS AND METHODS

Animal Models

All animal studies were conducted with prior approval from the Institutional Animal Care and Use Committee at the University of California, San Diego, and in accordance with guidelines established by the National Institutes for Health. *Preconditioning of pulmonary epithelium:* Male Balb/c mice were (8–12 week-old) preconditioned by isoflurane inhalation as described by Li et al [52] and lungs harvested (n = 6) after cervical dislocation. In control animals without preconditioning, mice (n = 6) were subjected to cervical dislocation without isoflurane anesthesia. *Laparatomy model:* 8–12-week-old male Balb/c mice (n = 6) were provided isoflurane anesthesia as above, the surgical site trimmed of hair, and prepared in a routine aseptic fashion. After verifying adequate anesthesia, the experimental animals received a 3 cm full-thickness midline incision deep to the peritoneum to expose the peritoneal cavity. Abdominal contents and bowel were manipulated for 15 seconds using the blunt end of sterile forceps to mimic an exploratory laparotomy. For closure, 5–0 running silk suture was used to approximate the edges of the abdominal wall, and six 5–0 interrupted silk sutures approximated the overlying skin. Animals were housed in separate cages with a 12 hour light/dark cycle and given access to food and water. Tissues were harvested at various time-points after surgery by cervical dislocation under isoflurane anesthesia. *Lipopolysaccharide (LPS) model of inflammation:* Male Sprague-Dawley rats (200—250 g) were randomly assigned to sham/control (n = 4), or LPS (n = 6) groups and LPS (15 mg/kg, *E. coli* Sigma L2654) in sterile saline was injected intravenously as previously described [53]. Lungs were harvested 6 hours after the injection of LPS.

Human Small Airway Epithelial (SAE) Cells in Culture

Human SAE cells from a 57-year-old female donor were purchased from Lonza Biologics (Walkersville, MD) at passage 2 and maintained in culture for less than 6 more passages as prescribed by the manufacturer. Cell lysates were prepared as described previously [26] but using SAE cells grown to 80% confluence. The ECRG4 protein was detected by

immunoblotting after SDS PAGE electrophoresis and gene expression by RT-PCR. Where indicated, cells were pretreated with 0.5 or 1 ug/mL LPS for 6 hours prior to measuring ECRG4. To assess the effects of ECRG4 on proliferation, cells were transduced with either control lentivirus (Vector Laboratories) or ECRG4 lentivirus and the transduced cells selected by flow activated cell sorting at UCSD core laboratories using expression of the bi-cistronic green fluorescent protein in the lentiviral vector. The sorted GFP^+ cells were distributed onto tissue culture plates for expansion and photographed (10×) 72 hours later.

Antibodies and Immunohistochemistry

Polyclonal antibodies were generated by contract with Genway (San Diego, CA) and raised in chickens by immunization using the sequence ECRG4 (71–148). Antibodies were immunoaffinity-purified with antigen and pre-immune IgY was used for controls. Lungs were collected at the times indicated and immediately fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS), pH 7.4. Samples were embedded in paraffin blocks for immunostaining and stored at room temperature prior to sectioning. At the time of immunohistological staining, paraffin sections were washed in xylene and ethanol to remove paraffin and incubated in citrate antigen retrieval solution (Thermo Scientific, Fremont, CA) for 30 minutes at 95°C. Sections were blocked with 10% donkey serum albumin in PBS for 1 hour and incubated overnight at 4°C in 0.5 μg/mL chicken anti-ECRG4 IgY. Sections were washed and incubated with 1 μg/mL biotin-conjugated donkey anti-chicken secondary antibody (Vector Laboratories) for 30 minutes at room temperature. Endogenous peroxidase activity was quenched in 0.3% H_2O_2 in methanol for 20 minutes before treatment with an avidinbiotin immunoperoxidase complex (ABC) (Vectastatin, Burlingame, CA) for amplification. Sections were incubated with diaminobenzidine substrate for 10 minutes to detect immunoreactivity, then counterstained with hematoxylin and mounted with Vectamount Mounting Solution on coverslips.

Immunoblotting

To determine the molecular weight of the ECRG4 peptide product, mouse lung was harvested, immediately frozen on dry ice, and kept at −80°C until analyzed. Total protein was extracted from mouse tissue by homogenization and sonication in 4× reducing lithium dodecyl sulfate (LDS) buffer (Invitrogen). Protein was size-fractionated by polyacrylamide gel electrophoresis (PAGE) on a 4–12% bis-tris gradient gel run in 2-(N-morpholino) ethanesulfonic acid (MES) buffer and transferred to a polyvinylidene fluoride membrane. Affinity purified anti-ECRG4 antibody (1:20,000) was used to detect ECRG4 while a horseradish peroxidase (HRP) conjugated goat anti-chicken antibody (BioRad) was used as the secondary antibody in immunoblots. An IVIS® Lumina imaging system (Caliper Life Sciences, Hopkinton, MA, USA) was used to detect signal generated by the horseradish peroxidase chromogenic substrate, DAB.

Real-time Quantitative PCR

Lungs were harvested at the indicated time points following injury or 6 hours after LPS injection, washed with normal saline, immediately preserved in RNAlater (QIAGEN Inc. Valencia, CA, USA) and stored at −80°C. At the time of analyses, the lungs were homogenized using FastPrep120 (Bio101 Savant) and total RNA extracted and mRNA

purified using the RNeasy mini kit (Qiagen, Valencia, CA, USA). Total mRNA was quantified by Nanodrop 2000 (Thermo Scientific Instruments, USA) and 1 μg was reverse-transcribed using the iScript cDNA synthesis kit (BioRad, Hercules, CA, USA). This cDNA was used to quantify the gene expression by real-time PCR (BioRad; iQ5 cycler) in a 25-μL reaction containing 100 nM each of sense and antisense primers for mouse ECRG4 (Qiagen, Catalog #QT00128051) and iQ SYBR Green Supermix (BioRad). The amplification cycle threshold (Ct) for ECRG4 was normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in each sample and either the delta–delta Ct ($2e^{-\Delta\Delta Ct}$) method or the plasmid standard curve was used to calculate fold-change in gene expression. Data represent the mean and standard deviation of 4–6 replicates as described in the legends to the figures. Statistical significance was evaluated by ANOVA and a $P < .05$ was considered significant.

RESULTS

ECRG4 mRNA and its Full-length Encoded Peptide Product are Detected in Mouse Lung

To assess the presence of ECRG4 in lung, we analyzed healthy mouse lung lysates for ECRG4 mRNA and its encoded peptide product. We performed qRT-PCR to measure ECRG4 expression in lung, and to compare several well-characterized pro-inflammatory cytokines associated with lung injury including TNF-α, IL-1β, IL-10, and IL-6 [54]. ECRG4 mRNA was present in lung and was expressed at levels greater than the referenced cytokines suggesting potential physiological significance of ECRG4 to lung biology (Figure 1A). Likewise, we performed qRT-PCR and demonstrated elevated ECRG4 lung expression (lane 1) in comparison to heart (lane 2) and skin (lane 3) (Figure 1B). Immunoblotting of mouse lung with the anti-ECRG4 antibody [27, 46] revealed the presence of a single 14 kDa peptide that corresponded to the molecular weight of the predicted precursor product, amino acids 31–148, of ECRG4 (Figure 1C).

ECRG4 is Localized to Mouse Type I Alveolar Cells

Immunohistochemical analysis of mouse lung revealed that ECRG4 is localized throughout the lung and at the cellular level is primarily associated with lung epithelial cells (Figure 2A). At high magnification, the ECRG4 positive cells were identified as type I alveolar epithelial cells (arrows in Figure 2B) based on their quantity and morphological features. In large part, fibroblasts and more cuboidal type ll alveolar cells appeared unstained. Immunostaining with isotype-matched control antibodies was performed to demonstrate the specificity of staining (Figure 2 insets) and minimal signal was detected.

ECRG4 Expression is Upregulated Following Preconditioning With Isoflurane

Increased ECRG4 expression in mucosal epithelium is protective in inflammation [29]. To test the possibility that lung ECRG4 might also be protective, we evaluated the ability of isoflurane preconditioning to alter ECRG4 expression because inhalation of isoflurane has been previously demonstrated to be protective in lung inflammation [55]. As shown in Figure 3, exposure to isoflurane caused a time-dependent increase in ECRG4 expression suggesting that the protective effects of isoflurane might be mediated, in part, by its capacity to upregulate ECRG4.

ECRG4 Expression is Downregulated Following Inflammatory Lung Injury

We examined the expression of ECRG4 mRNA after trauma with isoflurane anesthesia preconditioning to determine whether ECRG4 responds to trauma-induced lung injury. As shown in Figure 4A, exploratory laparotomy induced the expected inflammatory cytokine response in lung and a time dependent upregulation of TNF-α, IL-1β, IL-6, and IL-10 within 6 hours of surgery ($P < .05$). In contrast, *ECRG4* gene expression decreased eight-fold in this same time frame reaching a nadir expression at 6 hours post-treatment (Figure 4B). There was a subsequent recovery of *ECRG4* gene expression to pre-injury expression levels over the following 30 hours. Next, we tested ECRG4 expression following a second model of lung injury using intravenous injection of lipopolysaccharide (LPS) in rats. LPS-induced acute lung injury is a well described and characterized phenomenon that affects pulmonary function at the airway epithelium barrier permeability level [56]. Intravenous injection of LPS resulted in a similar downregulation of lung ECRG4. A three-fold decrease in ECRG4 expression (Figure 5) was detected 6 hours after LPS administration ($P < .03$).

ECRG4 Overexpression Inhibits Human Lung Epithelial Cells

Given the lack of feasible methods to directly test the effects of ECRG4 on type 1 alveolar epithelial cells in vitro, we turned to the availability of small airway epithelial cells where ECRG4 is also expressed. Detection of ECRG4 mRNA in human SAE cells in culture (Figure 6A) revealed low, but nevertheless detectable levels of ECRG4 expression. In addition, immunoblotting with antibodies against ECRG4 (Figure 6B, lane 1) detected the presence of the intact 14 kDa ECRG4 protein in SAE cell lysates. The addition of 1 μg/mL of LPS to the SAE cells for 6 hours (Figure 6B, lane 3) decreased the amounts of ECRG4 detected by immunoblotting by approximately 50%. ECRG4 overexpression in SAE cells appears growth inhibitory when SAE cells were transduced with either GFP-lentivirus or GFP-lentivirus that also encoded ECRG4. The transduced cells were sorted using GFP fluorescence (Figure 6C) and the cells engineered to express ECRG4 failed to proliferate for further passage (Figure 6D).

DISCUSSION

In this study, we show that ECRG4 mRNA and its encoded protein are normal constituents of type I alveolar epithelial cells in healthy mouse lung (Figures 1 and 2). We also show that there is increased *ECRG4* gene expression after protective preconditioning with isoflurane [55] and decreased *ECRG4* gene expression with lung injury (Figures 3–5). A kinetic analysis of this response shows that the decrease in ECRG4 expression after acute lung injury rebounds to pre-injury expression levels over time (Figure 4). Finally, the capacity of ECRG4 overexpression to block small airway lung epithelial cell proliferation in vitro (Figure 5) suggests that its downregulation in lung after injury in vivo might help enable a proliferative inflammatory response after injury. Similarly, its upregulation with preconditioning suggests that overexpression could be protective. Taken together, these results support our previous observations that epithelial cell-derived ECRG4 serves a sentinel function that gauges epithelial responsiveness, maintains homeostasis in healthy tissues and modulates the host response to injury.

In several models of injury and infection, when the decrease in ECRG4 expression is prevented by over-expression of ECRG4 prior to injury, the natural course of inflammation is significantly modified [27, 29, 46, 49]. Although a similar intervention was not performed here, these findings imply that the decrease in pulmonary ECRG4 observed after lung injury could equally translate to a permissive event that enables alveolar epithelial cell responsiveness (Figure 7).

Infection, inflammation, and trauma induce acute lung injury responses that include increased vascular permeability, decreased pulmonary function, inhibited alveolar gas exchange, and an activation of epithelial–mesenchymal transition (EMT) of type II alveolar epithelial cells [2, 57]. This EMT response is thought to contribute to the pathology of various acute and chronic pulmonary diseases that range from acute lung injury, to idiopathic pulmonary fibrosis and cancer. To this end, it is noteworthy that while there is a central role for TGF-β and β-catenin signaling, the loss of constitutive tumor suppressor gene expression (as shown here with ECRG4) could play an equally important, but dysinhibitory function, enabling EMT. If so, this could explain the relatively high levels of constitutive tumor suppressor gene expression in lung, compared to other cytokines (see Figure 1), point to a protective effect of the increased ECRG4 after preconditioning (see Figure 3) and suggest a physiologic consequence for its epigenetic regulation. Previous studies have clearly established the involvement of EMT in inflammatory injury and the pathogenesis of restrictive lung diseases [58, 59].

We developed a model implicating a physiological function for ECRG4 in lung injury and preconditioning (Figure 7). It presumes that constitutive homeostasis of type I and type II AECs (Figure 7A) is maintained at least in part, by epigenetic and environmental preconditioning of inhibitory molecule gene expression like *ECRG4*. First, ECRG4 encodes an inhibitory cell surface cytokine-like 14 kDa protein [26]. ECRG4 presumably plays an antiinflammatory inhibitory function that constitutively inhibits cell proliferation and by extension, blocks the EMT phenotype of type II AECs. Upon injury, however (Figure 7B), the release of local proteases and then activation and processing of ECRG4 [60], generates pro-inflammatory small peptides [25, 32, 49] that recruit inflammatory $CD16^{+}$ neutrophils and release cytokines that lead the inflammatory response. Downregulation of *ECRG4* gene expression prevents replacement of ECRG4 to the cell surface and a local dysinhibition of EMT enables proliferation (until such time that *ECRG4* gene expression re-emerges when proteases subside). In this model, methylation of the ECRG4 promoter gauges injury resolution by regulating the return of ECRG4 expression.

ECRG4 is normally expressed in healthy tissues and it is interesting to note that quiescent and injured tissues respond differently to subsequent injury [61, 62]. With ECRG4 expression regulated, in part, through epigenetic mechanisms like DNA methylation [28, 31, 37–42], the capacity of its promoter to upregulate the reservoir of ECRG4 expression in host tissues could also have an important influence on re-establishing homeostatic set points after injury. The decrease in ECRG4 gene expression has clearly demonstrated physiological consequences. For example, ECRG4 has been shown to inhibit cell growth in cancer, the development of neural progenitor cells, and inflammation after epithelial infection [25–27, 29, 36, 38, 43, 44, 46, 50, 63, 64]. As a membrane-tethered protein that undergoes release

upon stimulation [26, 64], intact 14 kDa ECRG4 appears to serve dual paracrine and autocrine functions while proteolytically processed ECRG4 peptides can have pro-inflammatory functions when ECRG4 is activated [32, 49]. To this end, ECRG4 therefore joins other cell signaling molecules including cytokines, chemokines, fractalkines, and growth factors that are normally cell membrane-associated proteins but also released after cell surface processing.

This first report of ECRG4 in lung provides insight into its possible role in pulmonary homeostasis and in gauging the injury response. Its activation and release, however, may have additional significance to lung biology that remains to be elucidated. In as much as several recent studies have shown a downregulation of ECRG4 gene expression after injury/inflammation that implies a dysinhibition of cell proliferation in vivo [27, 29, 46, 50, 51], its prototypic epigenetic regulation equally implies that any return to basal *ECRG4* gene expression levels after injury depends on preconditioning epigenetic factors like environment, diet, and age. Interestingly, these are the same factors that influence outcomes from pulmonary injury and infection and are associated with asthma, for example. Accordingly, we suggest that *ECRG4* gene expression in lung is affected by preconditioning and may influences the host injury response to inflammatory pulmonary injury.

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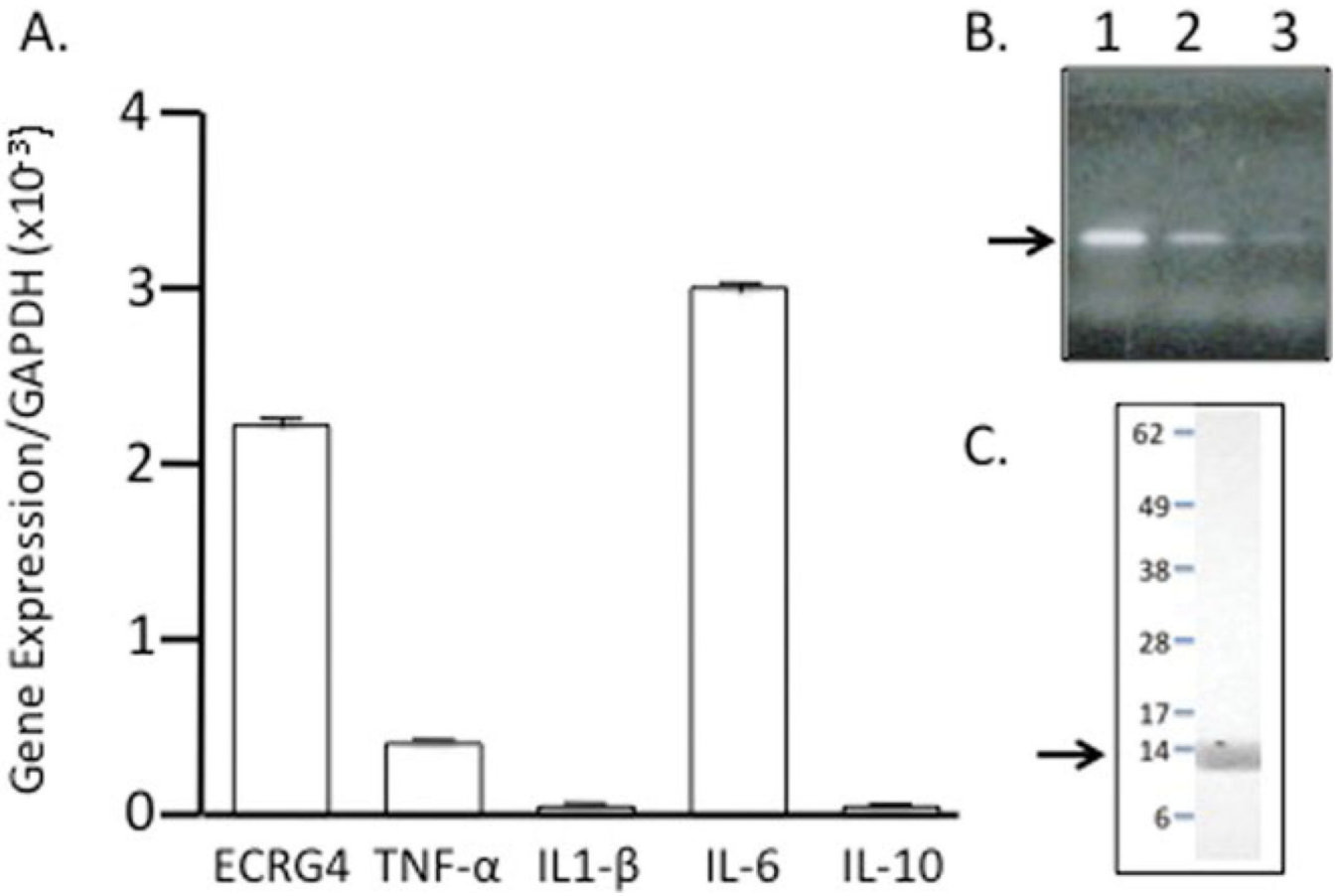

FIGURE 1.

ECRG4 is present in normal mouse lung. (A) Quantitative PCR was used to assess basal expression of ECRG4, TNF-α, IL-1β, IL-6, and IL-10 in healthy mouse lung. (B) RT-PCR of lysates from lung (lane 1), heart (lane 2), and skin (lane 3) show the expected amplified transcript fragment and suggest that ECRG4 is highly expressed in lung. (C) Immunoblotting revealed a 14 kDa ECRG4 protein in lung lysates. Molecular weight standards (kDa) are indicated. Results are presented as standard errors of the mean (SEM) and $P < .05$ as determined by ANOVA used as a measure of statistical significance.

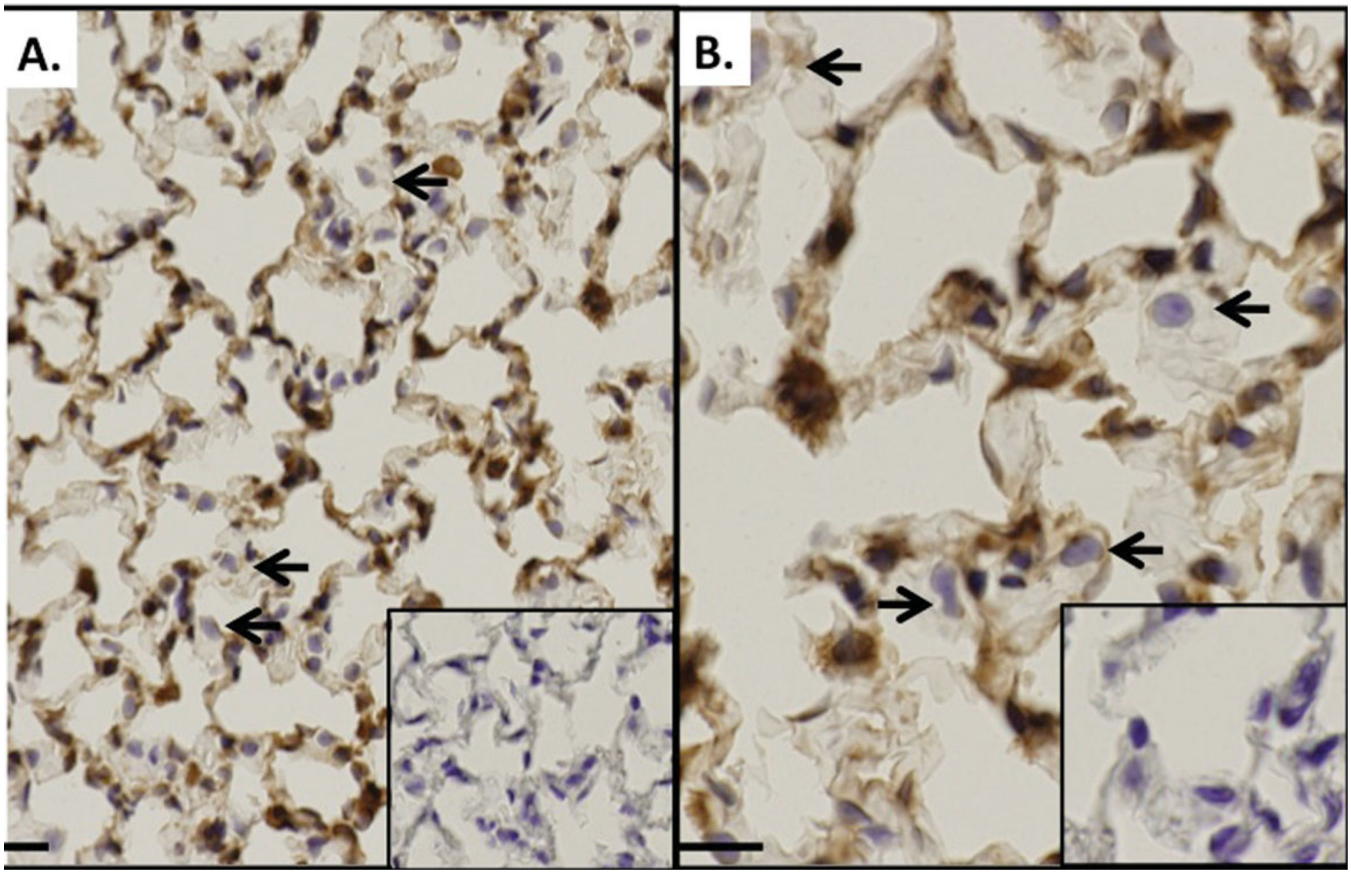

FIGURE 2.

Localization of ECRG4 to type I alveolar epithelial cells (A) Sections of paraffin-embedded normal mouse lung were immunostained with an anti-ECRG4 antibody and revealed distinct localization to thin, elongated cells that line the alveoli, consistent with type I alveolar epithelial cells. (B) Higher magnification photomicrograph demonstrates the absence of immunoreactivity in cells that appear morphologically consistent with type I alveolar epithelial pneumocytes. Minimal signal was detected in immunostaining (insets) with an isotype-matched control antibody, demonstrating antibody specificity. Size bar = 20 μm.

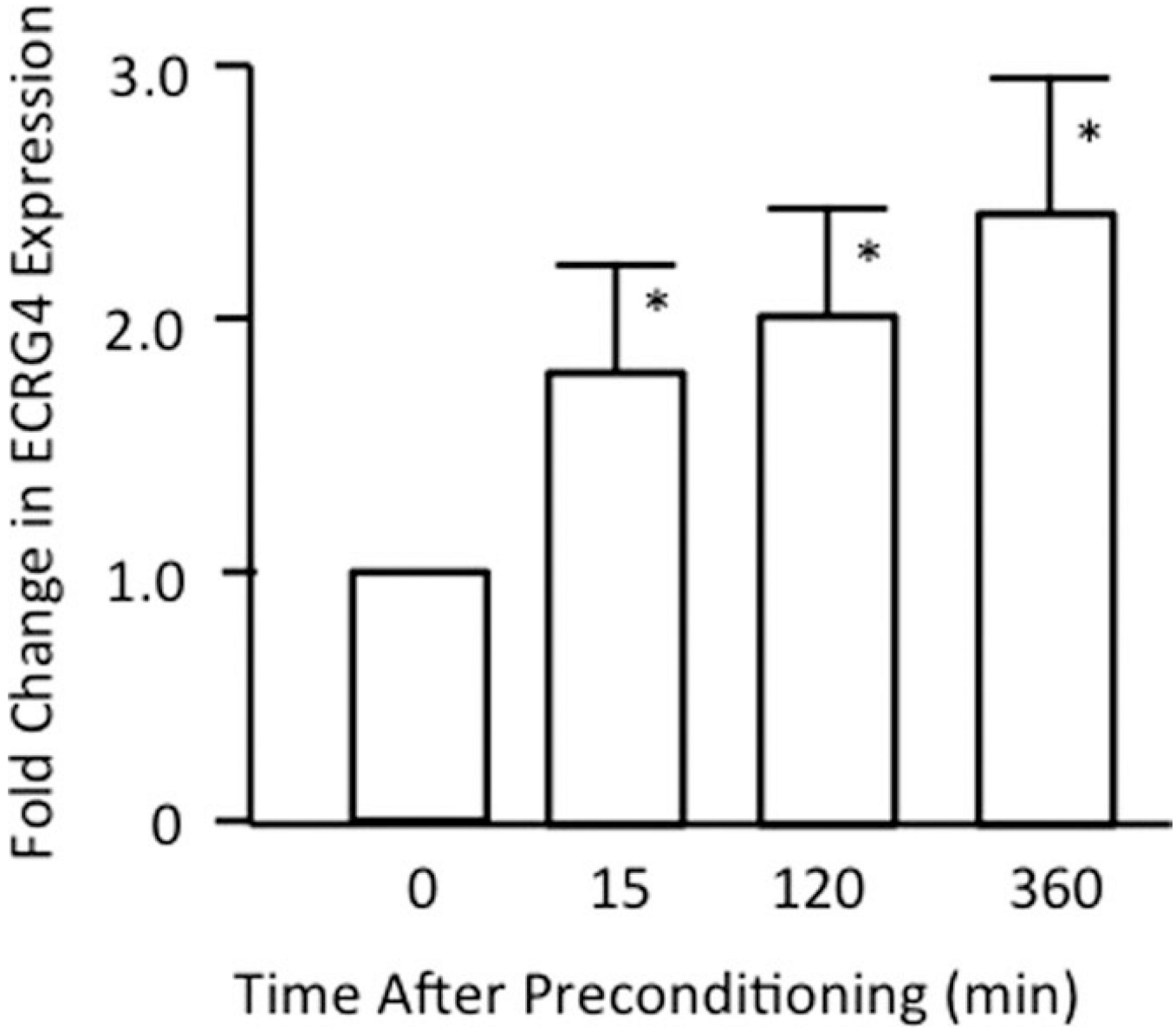

FIGURE 3.

Protective preconditioning increases ECRG4 Expression Male Balb/c mice (8–12 week-old) were preconditioned by isoflurane inhalation and lungs were harvested ($n = 6$) after cervical dislocation at the times indicated. Time 0 control mice were processed without preconditioning ($n = 6$) and subjected to cervical dislocation without isoflurane anesthesia. Quantitative PCR reveals an increase in *ECRG4* gene expression in the mouse lung following isoflurane inhalation. Results are presented as standard errors of the mean (SEM) and $P < .05$ as determined by ANOVA used as a measure of statistical significance.

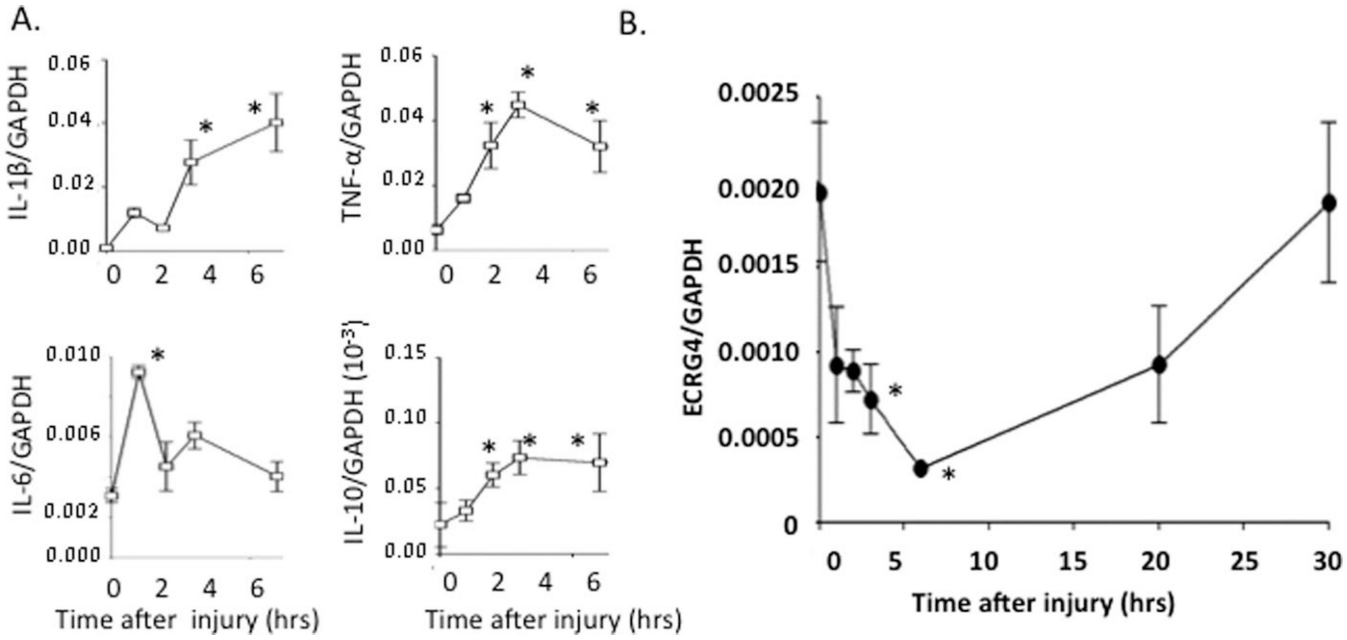

FIGURE 4.

Exploratory laparotomy induces transient ECRG4 downregulation along with upregulation of inflammatory cytokines in a mouse model of injury (A) Exploratory laparotomy induced an inflammatory lung injury response. Quantitative real-time PCR of lung at various time-points after exploratory laparotomy revealed the upregulation of TNF-α, IL1-β, IL-6, and IL-10 in lung within the acute 6 hour post-surgical period ($P < 0.05$, $n = 6$). (B) *ECRG4* gene expression kinetics following exploratory laparotomy. ECRG4 expression reached nadir levels 6 hours after exploratory laparotomy ($P < 0.05$) and rebounded to pre-injury expression levels with injury resolution at 30 hours ($n = 6$). Results are presented as standard errors of the mean (SEM) and $P < .05$ as determined by ANOVA used as a measure of statistical significance.

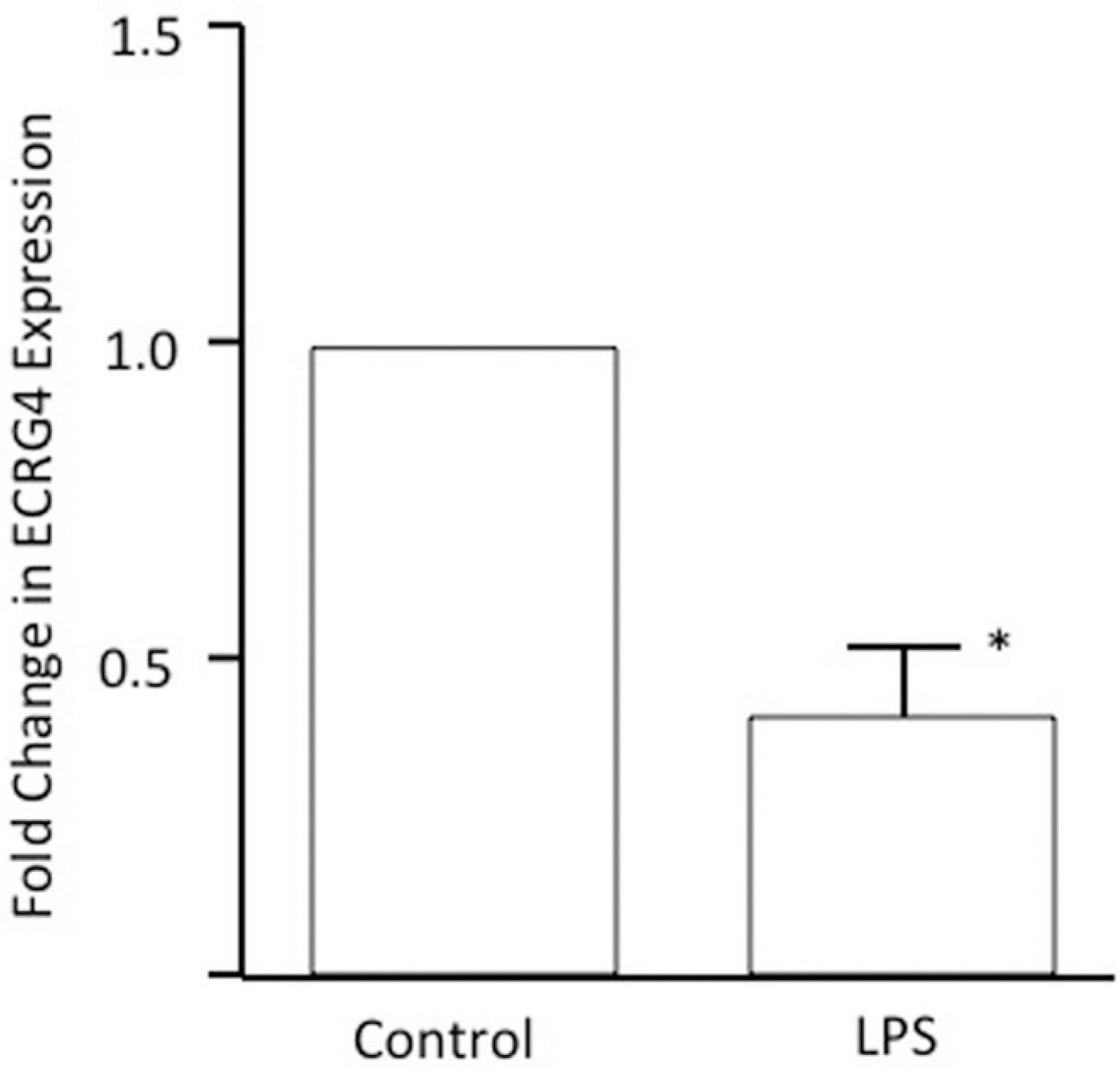

FIGURE 5.

LPS induces transient downregulation of ECRG4 in a rat model of sepsis. Lungs were harvested from male Sprague Dawley rats (200–250 g) 6 hours after random assignment and tail vein injection with either saline (control, $n = 4$) or LPS (15 mg/kg, $n = 6$). Tissues were harvested for gene expression analyses ($P < .05$, $n = 6$). Results are presented as standard errors of the mean (SEM) and $P < .05$ as determined by ANOVA used as a measure of statistical significance.

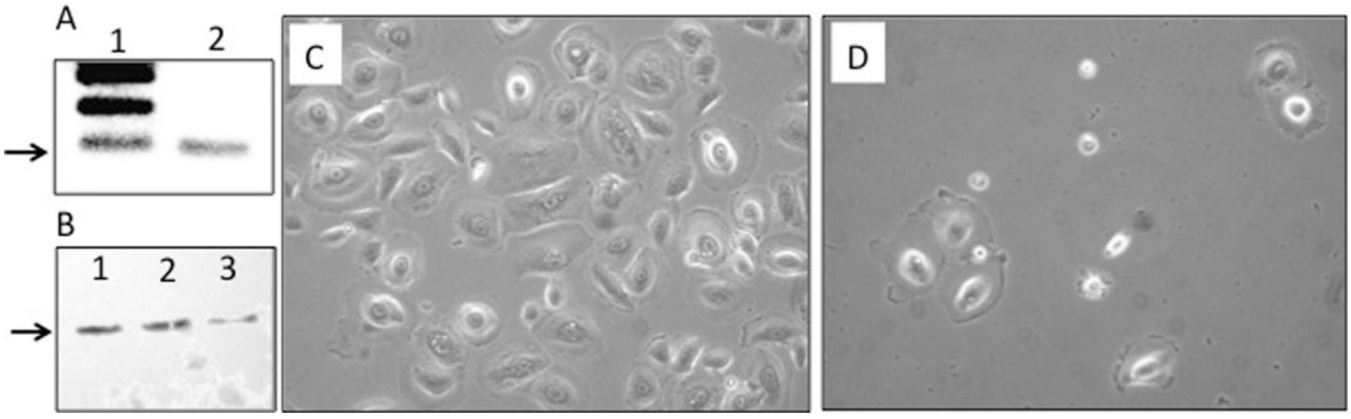

FIGURE 6.

ECRG4 is Present in Human Lung Epithelial Cells (A) Human small airway epithelial cells were extracted and RT-PCR used to detect *ECRG4* gene expression (lane 1 molecular weight standards, 100 bp). The expected ECRG4 transcript amplified (78 bp) is shown in lane 2. (B) Immunoblotting of human small airway epithelial cell lysates with anti-ECRG4 revealed a 14 kDa ECRG4 protein (lane 1) that was decreased by incubating cells for 6 hours with 0.5 (Lane 2) or 1 μg/mL (lane 3) of LPS. (C) When cells were transduced with control (Panel C) lentivirus or to overexpress ECRG4 (Panel D), the GFP-sorted cells that overexpressed ECRG4 had impaired growth capacity and failed to proliferate adequately for further passage.

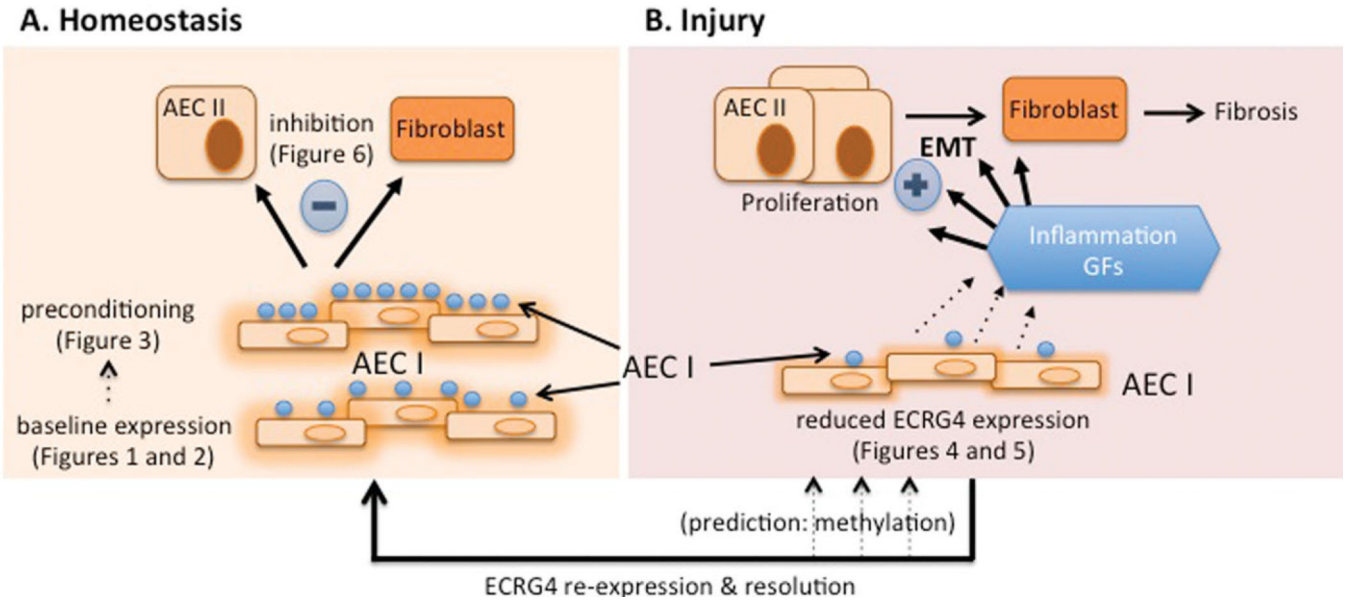

FIGURE 7.

A model for ECRG4 function in lung Epithelial to Mesenchyme Transition. (A) Normal type I alveolar epithelial cells constitutively express ECRG4 in a setting of minimal proliferation. ECRG4 serves a constitutive growth inhibitory function that can be upregulated by preconditioning. (B) Upon injury, infection and inflammation activation, there is a decrease of ECRG4 expression that coincides with recruitment of cytokines, inflammatory cells, and increased proliferation and cell motility. The resolution of inflammation is accompanied by a return of ECRG4; the scope of which is defined by preconditioning and the epigenetic methylation of its promoter.