Insulin-like growth factor-I stimulates differentiation of ATII cells to ATI-like cells through activation of Wnt5a

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Ghosh MC, Gorantla V, Makena PS, Luellen C, Sinclair SE, Schwingshackl A, Waters CM. Insulin-like growth factor-I stimulates differentiation of ATII cells to ATI-like cells through activation of Wnt5a. Am J Physiol Lung Cell Mol Physiol 305: L222–L228, 2013. First published May 24, 2013; doi:10.1152/ajplung.00014.2013.—Alveolar type II (ATII) cells are facultative progenitor cells that play a crucial role in the repair and remodeling of the lung following injury. ATII cells have the capability to proliferate and differentiate into alveolar type I (ATI) cells in vivo and into an ATI-like phenotype in vitro. While previous reports indicate that the differentiation of ATII cells into ATI cells is a complex biological process, the underlying mechanism responsible for differentiation is not fully understood. To investigate factors involved in this differentiation in culture, we used a PCR array and identified several genes that were either up- or downregulated in ATI-like cells (day 6 in culture) compared with day 2 ATII cells. Insulin-like growth factor-I (IGF-I) mRNA was increased nearly eightfold. We found that IGF-I was increased in the culture media of ATII-like cells demonstrating a significant role in the differentiation process. Treatment of ATII cells with recombinant IGF-I accelerated the differentiation process, and this effect was abrogated by the IGF-I receptor blocker PQ401. We found that Wnt5a, a member of the Wnt-Frizzled pathway, was activated during IGF-I-mediated differentiation. Both protein kinase C and β-catenin were transiently activated during transdifferentiation. Knocking down Wnt5a using small interfering RNA abrogated the differentiation process as indicated by changes in the expression of an ATII cell marker (prosurfactant protein-C). Treatment of wounded cells with either IGF-I or Wnt5a stimulated wound closure. These results suggest that IGF-I promotes differentiation of ATII to ATI cells through the activation of a noncanonical Wnt pathway.

ALVEOLAR TYPE II (ATII) cells are facultative progenitor cells that play a crucial role in the repair of the lung epithelium following injury (9, 32). Following lung injury, ATII cells spread, migrate, proliferate, and differentiate into alveolar type I (ATI) cells to cover the denuded surface. The underlying mechanism of this differentiation process is not fully understood (1, 8, 17, 45), but some aspects of this process are recapitulated in the differentiation of cultured ATII cells to ATI-like cells (3, 4, 10, 15, 20, 21). The microenvironment of the injured alveolus includes many cytokines, chemokines, and growth factors secreted from injured tissues, immune cells, and nonimmune cells (9). One of these components, insulin-like growth factor-I (IGF-I), has previously been shown to be involved in epithelial differentiation in the lungs (31, 35, 37). Alveolar epithelial cells and resident macrophages are the major two cells producing IGF-I in lung (27, 31, 37, 44, 46). Mice deficient in either IGF-I or its receptor exhibited lung hypoplasia and high mortality rates associated with respiratory failure (2, 38). Embryonic lungs of mice with deletion of the IGF-I gene had an increased proportion of ATI cells and less differentiated ATII cells (35). ATI cells isolated from hyperoxia-treated lungs transiently expressed increased IGF-I during transdifferentiation into type I-like cells (37). Although these studies suggest an important role for IGF-I in the differentiation of ATI cells, the underlying mechanism is not fully understood.

In the current study, we investigated the role of IGF-I in the differentiation of cultured rat ATII. Consistent with previous reports, we observed that ATII cells differentiate to ATI-like cells after 6 days of culture, and IGF-I expression was increased in ATI-like cells. We hypothesized that IGF-I-mediated signaling in ATII cell differentiation occurs through the Wnt-Frizzled (Wnt-Fzd) pathway based upon previous reports describing an association of the Wnt-Fzd system with IGF-I during organ development (39, 42). In addition, Wnt/β-catenin signaling has been associated with aberrant epithelial-mesenchymal transition in idiopathic pulmonary fibrosis (6, 32, 34) and an experimental model of emphysema (31). Targeted expression of an activated form of β-catenin caused ectopic differentiation of ATII cells in conducting airways (36). We found that IGF-I activates Wnt5a, which regulates the differentiation of ATII cells into ATI-like cells.

MATERIALS AND METHODS

Reagents. Dulbecco’s modified Eagle’s medium (DMEM), penicillin-streptomycin, trypsin-EDTA solution, and PBS were purchased from Gibco Life Technologies (Grand Island, NY). FBS was obtained from Hyclone (Logan, UT). HEPES, KCl, MgCl2, Triton X-100, sodium vanadate, phenylmethylsulfonyl fluoride, aprotinin, and leupeptin were purchased from Sigma (St. Louis, MO). Tween 20 was purchased from Bio-Rad (Herceus, CA). DRAQ5 (nuclear stain) was obtained from Molecular Probes (Eugene, OR). Rat IGF-I was purchased from Peprotech (Rocky Hill, NJ). The specific blocker for IGF receptor, PQ401, was purchased from Sigma Chemicals. Gradient gels (4–12%) for Western blot, and 10% gels, were purchased from Invitrogen (Carlsbad, CA). Antibodies for Wnt5a and Wnt3a were purchased from R & D (Minneapolis, MN). Control small interfering RNA (siRNA) and Wnt5a siRNA were purchased from Dharmacon (Lafayette, CO). Anti-rat antibodies for prosurfactant protein C (pro-SPC), a marker for ATII cells, were purchased from Millipore (Billericia, MA). Antibodies for rT140 and rTi170, which are...
markers for rat ATI and ATII cells, respectively, were generous gifts from Dr. Leland Dobbs, University of California-San Francisco. Antibodies for pan-protein kinase C (PKC) and β-catenin were purchased from abCAM (Cambridge, MA) and Cell Signaling Technology (Denver, CO), respectively.

**Cell culture.** Primary rat ATII cells were isolated according to the methods described previously (13, 14). The animal protocol was approved by the Institutional Animal Care and Use Committee at the University of Tennessee Health Science Center. Briefly, ATII cells were isolated from male Sprague-Dawley rats by elastase digestion and differential adherence on IgG-coated dishes. ATII cells were identified using Nile Red (Sigma-Aldrich) staining of lamellar bodies, and >95% of the cells were Nile Red-positive on day 2. We used plastic six-well plates that were coated with rat lung fibroblast (RLF) matrix deposited by RLF-6 cells (American Type Culture Collection) for culture of ATII cells (13). Freshly isolated cells were seeded to confluence at 3.5 × 10^5 cells/well in ATII culture medium (DMEM with 10% FBS, 4 mM glutamine, 1% penicillin/streptomycin, and 0.25 μM amphotericin B), and experiments were performed on day 2 after isolation. To obtain ATI-like cells, ATII cells were cultured until day 6 from the day of isolation with changing of the media every day. On day 2 or day 6, cells were harvested, and lysate was made. Lysate was stored at −70°C for future use. Media were collected and stored at −70°C for estimation of IGF-I. A549 cells and MLE-12 cells were cultured in DMEM with 10% FBS and 0.5% penicillin/streptomycin.

**Treatment of ATII cell.** Cells were treated with IGF-I at 50 ng/ml as indicated. PQ401 (IGF receptor blocker) was used at a dose of 2 μg/ml. The incubation times for both IGF-I and blocker were 24 h wherever applicable. There was no toxicity or cell death observed by Trypan blue exclusion test after 24 h of incubation for either of those reagents. For combined treatment, cells were treated with PQ401 for 2 h before addition of IGF-I.

**PCR array.** A 96-well-based quantitative real-time PCR Array kit that targets rat stem cell-associated genes was purchased from SA-Biosciences (Valencia, CA) (catalog no. PARN-405). Total RNA was isolated from ATII and ATI-like cells followed by conversion of RNA to cDNA using a kit (Bio-Rad). Equal amounts of RNA were used (500 ng) from ATII and ATI-like cells to make cDNA. Real-time PCR array reagents were purchased from SA-Bioscience. Raw data of real-time PCR were analyzed by using software provided by SA-Bioscience.

**Luminex assay.** Phosphorylation of insulin receptor substrate-1 was examined by running a Luminex-based assay kit purchased from Millipore (catalog no. 48–61). ATII cells were treated with IGF-I for different time points, and lysate was made using the lysis buffer supplied in the kit. Equal amounts of protein (50 μg) were used for the assay. All the steps were performed according to instructions of the manufacturer.

**ELISA of IGF-I.** The amount of secreted IGF-I was measured from media of ATII and ATI-like cultured cells using an ELISA kit purchased from Mediangost (Reutlingen, Germany) (catalog no. m/IGF-I ELISA). Media were collected when cells became confluent on the plates. Multiple scratch wounds were made on the confluent layer of cells following the method previously described (12, 19). The amount of IGF-I secreted from the cells after 24 h was determined.

**Wound healing assay.** Cell migration was measured according to our previous methods (12, 19). Confluent monolayers of ATII or MLE-12 cells were wounded by scraping a pipette tip across the monolayer to produce initial wounds of 1,000–1,200 μm. Images were collected with a Cool Snap charge-coupled device camera (Roper Scientific, Trenton, NJ) mounted on an Eclipse TE300 inverted microscope with a 4 × objective (Nikon, Melville, NY). Images were obtained at the initial time of wounding and then 24 h post-wounding. Metamorph software was used to record the coordinates for each wound location using a computer-controlled stage so that the same location was used for postwounding measurements, and data were analyzed by Metamorph imaging software. The mean wound width at 24 h was calculated and normalized to the original wound width. All results reported are based on at least three independent wells of two separate experiments (n = 6).

**SDS-PAGE.** Protein concentration was determined by the Bradford (5) method. Equal amounts of protein were electrophoretically separated by 4–12% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. Membranes were blocked for 1 h in 5% nonfat milk (Bio-Rad) in Tris-buffered saline containing 0.01% Tween 20 (TBST) and incubated overnight at 4°C with the appropriate primary antibody. Membranes were washed with TBST and then incubated with the secondary antibody for 1 h at room temperature. Finally, blots were developed on X-ray film using the Luminata method (Millipore).

**Transient knockdown of Wnt5a in ATII cells.** Wnt5a was transiently knocked down in ATII cells using siRNA purchased from Dharmacon. Subconfluent (50–60%) ATII cells were transfected with Wnt5a siRNA or control siRNA using the siRNA transfection kit (Santa Cruz, CA). After 48 h of transfection, cells were harvested, and lysate was made for immunoblot.

**Confocal microscopy.** Expression of pro-SPC in ATII cells was assessed by confocal microscopy. ATII cells transfected with Wnt5a or control siRNA were fixed with 4% paraformaldehyde. Cells were permeabilized with 0.1% Triton X-100 followed by blocking with 2% BSA. Pro-SPC primary antibody was incubated at 1:100 dilution overnight at 4°C. The secondary antibody labeled with Alexa fluor-488 (Molecular Probes, Eugene, OR) was used at 1:500 dilution at room temperature for 30 min. The nucleus was stained with DRAQ-5 (Molecular Probes) at 1:1,000 dilution for 15 min at room temperature. Images were obtained using a confocal microscope (Olympus) using a ×20 objective.

**Densitometry of immunoblot.** Densitometry of bands obtained by immunoblot was performed using the software Image-J developed by the National Institutes of Health. Each band was normalized against the corresponding loading control, β-actin, or GAPDH.

**Quantification of immunofluorescence.** Immunofluorescence was quantified using the mean fluorescence intensity determined using Image-J software. Mean fluorescence intensity was measured from five different fields that were randomly selected.

**Statistical analysis.** All values are presented as means ± SE. Each value represented the mean of at least three independent experiments with SE. A t-test was used when only two groups were compared, and one-way ANOVA with the Holm-Sidak method was performed for comparisons of multiple treatments to determine significant differences between individual conditions. Significant differences were determined based on a threshold of P < 0.05. Statistical comparisons were made using SigmaStat 3.5 (Jandel Scientific, San Rafael, CA).

**RESULTS**

**ATII cells differentiate to ATI-like cells in vitro.** To confirm previous findings that ATII cells differentiate into ATI-like cells in culture, we compared ATII cells isolated from rats after 2 or 6 days of culture. The ATII surface marker rTI70 was significantly decreased after 6 days in culture compared with day 2 cells (Fig. 1A), whereas the ATII surface marker rTI40 was significantly higher (P < 0.01) on the 6th day compared with day 2 ATII cells (Fig. 1B).

**IGF-I was upregulated during differentiation of ATII cells.** To gain insight into the mechanisms of this differentiation, we used a PCR-based array that targeted the genes of rat stem cell differentiation to compare day 2 ATII cells with day 6 ATI-like cells. As shown in Fig. 2A, at least nine genes were upregulated, and three genes were downregulated more than twofold. Among the upregulated genes, IGF-I was increased approximately eightfold in ATI-like cells compared with ATII cells. To determine whether secretion of IGF-I was increased in
ATII-like cells, we measured the IGF-I from conditioned media. Secretion of IGF-I was significantly increased in ATII-like cells compared with ATII cells after 24 h (Fig. 2B). Interestingly, when we applied multiple scratch wounds to cell monolayers, there was a significant increase in IGF-I secretion in both ATII and ATII-like cells (Fig. 2B). To determine the responsiveness of ATII cells to IGF-I in our system, we measured the phosphorylation of insulin receptor substrate-1 (22) in response to exogenous IGF-I using the Luminex system. Treatment with IGF-I (50 ng/ml) caused a significant increase in phosphorylation of insulin receptor substrate-1 in ATII cells within 5 min that was sustained for at least 60 min (Fig. 2C).

Activation of Wnt5a and Wnt3a during transdifferentiation. Because the Wnt-Fzd system has previously been shown to be involved in epithelial cell differentiation, we investigated the expression of Wnt5a and Wnt3a in ATII cells as a function of time in culture. Wnt5a is part of the noncanonical Wnt pathway, whereas Wnt3a is part of the canonical pathway. Both Wnt5a and Wnt3a were upregulated during the course of differentiation in culture, but, while Wnt5a expression peaked by day 4 and remained elevated, Wnt3a expression peaked by day 3 and then declined (Fig. 3). In parallel, we examined the level of pro-SPC, an ATII cell marker during the course of differentiation. We observed that the amount of pro-SPC was high in the first 2 days in culture but decreased significantly by day 3 (Fig. 3, C and D). For comparison, we measured the expression of Wnt5a and pro-SPC in A549 cells following treatment with IGF-I for 72 h. Although the magnitude of the changes was not as high as in primary ATII cells, we found that treatment with IGF-I did cause a significant decrease in expression of pro-SPC and a significant increase in expression of Wnt5a (Fig. 3, E and F).

Upregulation of pan-PKC and β-catenin during transdifferentiation of ATII cells. Activation of Wnt5a and Wnt3a is associated with upregulation of PKC and β-catenin, respectively. Therefore, we investigated the status of these two proteins during differentiation of ATII cells to ATI cells. Immunoblots demonstrated that upregulation of both pan-PKC and β-catenin occurred during differentiation of ATII cells (Fig. 4). The maximal level was observed at day 2 for pan-PKC and day 3 for β-catenin followed by gradual decline in the expression of each.

Wnt5a upregulation was stimulated by IGF-I. To determine whether Wnt5a or Wnt3a induction was stimulated by IGF-I, we treated ATII cells from day 2 with IGF-I (50 ng/ml) for 24 h and immunoblotted for Wnt5a and Wnt3a. As shown in Fig. 4A, IGF-I treatment stimulated increased expression of Wnt5a (Fig. 5A) but not Wnt3a (Fig. 5B). When cells were pretreated with the IGF-I receptor blocker PQ401 before IGF-I, there was no increase in Wnt5a expression.
Fig. 3. Activation of Wnt3a and Wnt5a during transdifferentiation. Representative immunoblots of Wnt3a, Wnt5a, and prosurfactant protein-C (pro-SPC) from lysate from ATII cells cultured from 0 to 6 days are shown (A, B, and C, respectively). β-Actin was immunblotted as a loading control. D: densitometry for Wnt3a, Wnt5a, and Pro-SPC (n = 4). *Significant difference from day 0 (P < 0.05). E and F: representative immunoblots and densitometry of Wnt5a (n = 5) and pro-SPC (n = 3) in A549 cells treated with IGF-I for 72 h. *Significant difference from untreated control (P < 0.05).

Downregulation of Wnt5a delays transdifferentiation. To examine the role of Wnt5a in the differentiation of ATI cells, we knocked down Wnt5a using siRNA. Figure 6A shows that siRNA directed against Wnt5a caused a 50% reduction in expression compared with cells transfected with a control siRNA. To determine the effect of Wnt5a siRNA on transdifferentiation, we measured the expression of pro-SPC as a phenotypic marker of ATII cells. When ATII cells were transfected with control siRNA on day 1 and treated with IGF-I for 24 h beginning on day 2, there was a loss of pro-SPC expression (Fig. 6B). Cells with knockdown of Wnt5a showed markedly increased pro-SPC expression in the absence of IGF-I, but treatment with IGF-I reduced pro-SPC expression in these cells (Fig. 6B).

IGF-I and Wnt5a augment wound healing in MLE-12 cells. To determine whether Wnt5a expression was changed following injury, we measured the expression of Wnt5a in a cell line of mouse lung epithelial cells (MLE-12) exposed to multiple scratch wounds. Compared with unwounded cells, cells exposed to multiple scratch wounds had significantly increased Wnt5a expression after 36 h (Fig. 7A). We then investigated whether treatment with either IGF-I or Wnt5a would affect wound healing following a scratch wound. Figure 7B shows that wound closure was stimulated by treatment with either IGF-I, Wnt5a, or the combination of the two.

DISCUSSION

Epithelial repair following lung injury involves differentiation of ATI cells, but the mechanisms involved in ATI cell differentiation into ATI cells is not fully understood. Consistent with previous reports (3, 15, 20, 21), our data showed that ATI cells in culture differentiated into ATI-like cells over 2–6 days as indicated by specific markers rTII70 and rTI40 (Fig. 1). To investigate factors involved in this differentiation in culture, we used a PCR array targeted at stem cell differentiation and identified several genes that were either up- or downregulated in ATI-like cells (day 6 in culture) compared with day 2 ATI cells. IGF-I mRNA was increased nearly eightfold (Fig. 2A). We then measured IGF-I protein levels in the conditioned media and found a significant increase in IGF-I in ATI-like cells compared with control cells (Fig. 2B). Gonzalez et al. (20) had previously performed a comparison of freshly isolated ATI cells (day 0) and freshly isolated ATII cells (day 0) from rats. Using microarray analysis, they found a 5.5-fold increase in gene expression of IGF-binding protein 6 (IGFBP6) in ATI cells compared with ATII cells. IGFBP6 is expressed in the lung and regulates the bioavailability of IGF-I by binding with its active conformation (41). While our data and the microarray analysis of Gonzalez et al. (20) suggested differential expression of numerous other genes between ATII and ATI cells (and between ATII cells cultured for 6 days), these results support a
potential role for IGF-I in the differentiation of ATII cells to ATI cells.

Previous studies have suggested that IGF-I is elevated in the bronchoalveolar lavage fluid in patients with early acute respiratory distress syndrome (ARDS) (40), and patients with fibroproliferative ARDS exhibited increased IGF-I immunostaining in biopsy tissue sections (27). IGF-I mRNA was also elevated in a mouse model of bleomycin-induced pulmonary fibrosis (30), and the use of IGF-I receptor antibodies improved survival of mice with bleomycin-induced lung injury (7). Based upon these findings, it has been proposed that IGF-I may promote the survival of lung fibroblasts and subsequent fibrosis. However, an additional role of IGF-I may be the promotion of differentiation of ATII to ATI cells following injury. We found that IGF-I secretion was significantly increased in both ATII and ATI-like cells following a scratch wound injury (Fig. 2B). A previous report demonstrated that treatment of ATII cells with antibodies against IGF-I or the IGF I receptor inhibited transdifferentiation (as indicated by expression of ATII and ATI markers) when the cells were isolated from rats following exposure to hyperoxia in vivo (37). This study also found that mRNA expression for IGF-I, IGF-II, and the IGF-I receptor increased during exposure to hyperoxia in vivo, and protein levels remained elevated during 3 days of recovery and then decreased. Ionescu et al. (24) demonstrated that treatment with recombinant IGF-I reduced LPS-induced lung injury in mice and suggested that IGF-I may be secreted by mesenchymal stem cells during repair following injury. To investigate the signaling pathways initiated by IGF-I, we first examined the phosphorylation of insulin receptor substrate-1, which binds with the IGF-I receptor and becomes phosphorylated (22). Luminex data demonstrated the rapid phosphorylation of the insulin receptor substrate-1 upon treatment with IGF-I, indicating activation of the IGF-I receptor (Fig. 2C).
We then investigated the activation of the Wnt-Fzd system since previous studies have suggested that IGF-I can activate this system during organ development (39, 42). In addition, microarray comparison between freshly isolated ATII and ATI cells demonstrated a fourfold increase in expression of Fzd-2 in ATI cells relative to ATII cells (20). Surprisingly, we found that activation of both canonical (Wnt3a) and noncanonical (Wnt5a) Wnt pathways occurred in ATII cells with time in culture (Fig. 3). Wnt5a has previously been shown to antagonize canonical Wnt signaling in esophageal carcinoma cells and other cell lines (28, 34, 43), but, to our knowledge, this is the first report in primary ATII cells. We found that expression of both Wnt3a and Wnt5a increased until day 4 in culture at which time Wnt3a expression decreased and Wnt5a remained stable. Wnt3a signaling involves stabilization and activation of β-catenin, whereas Wnt5a signaling involves stimulation of intracellular Ca²⁺ release and activation of PKC (34). To provide additional support for the simultaneous activation of Wnt5a and Wnt3a, we examined the expression PKC and β-catenin (23, 29, 33, 47). We found that both were transiently upregulated during the differentiation of ATII to ATI-like cells (Fig. 4). We also found that treatment of A549 cells with IGF-I stimulated a decrease in pro-SPC expression and an increase in Wnt5a expression (Fig. 3).

Our results showing increased Wnt3a expression are consistent with the previous finding that β-catenin signaling was not present in freshly isolated ATII cells but increased by day 2 in culture (18). Forced inhibition of β-catenin signaling caused increased cell death and reduced expression of ATII cell markers by day 3 in culture. Flozak et al. (18) also demonstrated that adult ATI cells did not exhibit constitutive β-catenin signaling in vivo using the AXIN2+/−/LacZ reporter mouse, but β-catenin was activated in ATII cells following bleomycin injury. β-Catenin pathways are reported to be induced in idiopathic pulmonary fibrosis or bleomycin-induced lung fibrosis, which are associated with epithelial-to-mesenchymal transition of alveolar epithelial cells (6, 26). We found for the first time that the activation of Wnt5a was dependent upon IGF-I signaling, but activation of Wnt3a was not affected by IGF-I (Fig. 5). To further confirm the importance of Wnt5a in ATII cell transdifferentiation, we showed that siRNA knockdown of Wnt5a decreased expression of pro-SPC in control and IGF-I-treated ATII cells (Fig. 6).

Previous studies have suggested that IGF-I can stimulate wound repair in other organs (11, 16, 25). In preliminary studies, we found that treatment of rat ATII cells with IGF-I did not stimulate wound closure over 16 h in a scratch wound model (data not shown). It is possible that a longer exposure time is necessary to observe effects on wound closure, but we were concerned that our studies might be complicated by the changes taking place in the cells during transdifferentiation. Therefore, we examined the response in MLE-12 cells over 36 h. We found that both IGF-I and Wnt5a treatment stimulated wound closure in a scratch wound assay (Fig. 7).

Taken together, our results and the previous studies suggest that both Wnt3a and Wnt5a activation occurs in response to injury and upon culture of freshly isolated ATII cells to initiate differentiation into ATI cells. However, activation of Wnt5a may ultimately antagonize Wnt3a signaling while further promoting the transition. Our results suggest that secretion of IGF-I from ATII cells results in autocrine activation of Wnt5a signaling that promotes both wound repair and differentiation.


