ANIMAL MODELS

Adenosine A_{2a} Receptor Blockade Diminishes Wnt/ β -Catenin Signaling in a Murine Model of Bleomycin-Induced Dermal Fibrosis

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Adenosine A_{2a} receptor (A_{2a}R) stimulation promotes the synthesis of collagens I and III, and we have recently demonstrated that there is crosstalk between the A_{2a}R and WNT/ β -catenin signaling pathway. In *in vitro* studies, A_{2a}R signaling for collagen III expression was mediated by WNT/ β -catenin signaling in human dermal fibroblasts; we further verified whether the crosstalk between A_{2a}R and Wnt/ β -catenin signaling was involved in diffuse dermal fibrosis *in vivo*. Wnt-signaling reporter mice (*Tcf/Lef:H2B-GFP*) were challenged with bleomycin and treated with the selective A_{2a}R antagonist istradefylline (KW6002) or vehicle. Dermal fibrosis was quantitated and nuclear translocation of β -catenin in fibroblasts was assessed by double-staining for Green fluorescent protein or dephosphorylated β -catenin or β -catenin phosphorylated at Ser552, and vimentin. KW6002 significantly reduced skin thickness, skinfold thickness, breaking tension, dermal hydroxyproline content, myofibroblast accumulation, and collagen alignment in bleomycin-induced dermal fibrosis. Also, there was increased expression of *Tcf/Lef:H2B-GFP* reporter in bleomycin-induced dermal fibrosis, an effect that was diminished by treatment with KW6002. Moreover, KW6002 significantly inhibited nuclear translocation of *Tcf/Lef:H2B-GFP* reporter, as well as dephosphorylated β -catenin and β -catenin phosphorylated at Ser552. Our work supports the hypothesis that pharmacologic blockade of A_{2a}R inhibits the WNT/ β -catenin signaling pathway, contributing to its capacity to inhibit dermal fibrosis in diseases such as scleroderma. (*Am J Pathol* 2017, 187: 1935–1944; <http://dx.doi.org/10.1016/j.ajpath.2017.05.005>)

Dermal fibrosis is a pathologic hallmark of several disorders, including scleroderma, hypertrophic scarring, and keloid, and arises from excessive deposition of collagen and other extracellular matrix components released by pathologically activated fibroblasts.^{1,2} Despite a growing effort to understand and target the mechanisms involved in fibrosis, the choice and efficacy of treatment are limited, and the development of new antifibrotic therapies awaits the elucidation of better targets.³

Adenosine, a small molecule generated extracellularly from adenine nucleotides after direct cellular stimulation, hypoxia, injury, or metabolic stress,^{4,5} acts via a family of classic seven transmembrane G protein–coupled receptors (A₁, A_{2a}, A_{2b}, and A₃) that are expressed in a cell- and tissue-specific manner. Adenosine A_{2a} receptor (A_{2a}R), the subtype most relevant to dermal fibrosis, signals via G_s

proteins, which activate adenylyl cyclase, leading to an increase in intracellular cAMP. The rise in cAMP, in turn, activates downstream targets such as protein kinase A and the exchange factor directly activated by cAMP proteins 1/2, which results in fibroblast activation and collagen synthesis. Our previous studies demonstrated that A_{2a}R, but not A₁R or A_{2b}R, stimulates dermal fibroblasts to

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produce collagens I and III (COL1A1 and COL3A1) and down-regulates the expression of matrix metalloproteinases 2, 9, and 14, which are involved in collagen breakdown.⁶ Although the capacity to generate adenosine from ATP is necessary for bleomycin to induce dermal fibrosis,⁷ prior studies have also shown that signaling via A_{2a}R is required for bleomycin to induce fibrosis, as A_{2a}R knockout mice and A_{2a}R pharmacologic blockade in wild-type mice prevented bleomycin-induced dermal fibrosis⁶ and radiation-induced dermal injury.⁸ The inability to generate adenosine from ATP [due to deficiency of ecto-5'-nucleotidase (CD73) and ectonucleoside triphosphate diphosphohydrolase-1 (CD39)] also prevents bleomycin-induced dermal fibrosis.⁷ Moreover, deficiency in adenosine deaminase, the principal catabolic enzyme for adenosine *in vivo*, leads to elevated adenosine levels and spontaneous dermal fibrosis in mice, which is also significantly prevented by the administration of an A_{2a}R antagonist.⁹

The wingless-type mouse mammary tumor virus integration site family (WNT)/ β -catenin signaling pathway has been found to regulate cell-fate decisions during normal organ development and tissue homeostasis.¹⁰ Accumulating studies have demonstrated that an aberrant WNT/ β -catenin signaling pathway plays an important role in wound healing and fibrosis.^{11–13} In the absence of WNT signals, the β -catenin–destruction complex, which includes the key regulatory enzyme glycogen synthase kinase 3 β , constitutively phosphorylates β -catenin, which promotes subsequent degradation of β -catenin. Binding of WNTs to their frizzled receptors, however, disrupts the destruction complex, and thus prevents the degradation of β -catenin. Once stabilized, cytoplasmic β -catenin accumulates and subsequently translocates to the nucleus, where it binds to members of the T-cell factor/lymphoid enhancer factor (TCF/LEF) family of proteins and forms β -catenin–TCF/LEF transcription factor complexes to activate target genes,¹⁰ including profibrotic genes (eg, fibronectin, matrix metalloproteinase-7, plasminogen activator inhibitor-1, twist, and snail).¹³ Besides canonical activation, β -catenin is also phosphorylated on the indicated sites by diverse kinases such as protein kinase A, protein kinase B (AKT), and janus kinase 2, leading to β -catenin stabilization and the activation of downstream genes.¹⁴

We recently reported that A_{2a}R stimulation promotes COL3A1 synthesis via β -catenin activation in normal human dermal fibroblasts.¹⁵ We found that stimulation with the A_{2a}R agonist CGS21680 rapidly (within 15 minutes) increases cellular β -catenin, dephosphorylated β -catenin (de-phospho β -catenin; canonical β -catenin activation) and β -catenin phosphorylated at Ser552 (p-Ser552 β -catenin; noncanonical β -catenin activation, site of β -catenin activation by AKT) levels in normal human dermal fibroblasts, resulting in nuclear translocation of β -catenin, an effect blocked by the A_{2a}R selective antagonist SCH58261. β -Catenin knockdown abrogates the A_{2a}R-stimulated increments in COL3A1 synthesis, but not COL1A1 expression.¹⁵

This study aimed to further verify the crosstalk between A_{2a}R and Wnt signaling in the pathogenesis of dermal fibrosis *in vivo*. Using Wnt-signaling reporter mice (*Tcf/Lef:H2B-GFP*), we report on reporter gene expression in dermal fibroblasts in bleomycin-induced dermal fibrosis used in this murine model of scleroderma, and the impact of A_{2a}R antagonist istradefylline (KW6002) on this effect. Moreover, we report the effects of A_{2a}R pharmacologic blockade on nuclear translocation of *Tcf/Lef:H2B-GFP* reporter, de-phospho β -catenin, and p-Ser552 β -catenin.

Materials and Methods

Reagents

Bleomycin was purchased from Fresenius Kabi (Grand Island, NY). KW6002 was purchased from Tocris Bioscience (Ellisville, MO). Rabbit polyclonal antibody to de-phospho β -catenin (Ser33/37/Thr41) and p-Ser552 β -catenin were purchased from Cell Signaling Technology (Danvers, MA). Mouse monoclonal antibodies to vimentin and α -smooth muscle actin (α -SMA) were purchased from Abcam (Cambridge, MA). Rabbit monoclonal antibody to vimentin, DAPI mounting medium, goat anti-rabbit IgG (whole-molecule)—fluorescein isothiocyanate secondary antibody, bovine serum albumin, hydroxyproline, chloramine-T, and Ehrlich solution were purchased from Sigma-Aldrich (St. Louis, MO). Mouse monoclonal antibody to Green fluorescent protein (GFP), donkey anti-mouse IgG (H+L)—Alexa Fluor 555 conjugate secondary antibody, fetal bovine serum, and propanol were purchased from Thermo Fisher Scientific (Rochester, NY).

Animal Model

Wnt-signaling reporter mice (*Tcf/Lef:H2B-GFP*) were purchased from The Jackson Laboratory (Bar Harbor, ME). Transgene induction was performed as described previously.¹⁶ Male *Tcf/Lef:H2B-GFP* mice age 13 weeks (25 to 30 g) in this murine model of scleroderma were treated with the A_{2a}R antagonist KW6002 (10 mg/kg once per day i.p., administered in vehicle consisting of 8% Tween 80 in saline to a total injection volume of 10 μ L/kg) or vehicle alone, starting 3 days before dermal fibrosis induction with bleomycin (1.5 mg/mL, 0.1 mL s.c. in a 1.0-cm² area on the upper back, every other day for 3 weeks). Mice injected with 0.1 mL of phosphate buffered saline (PBS) s.c. were used as negative controls. Male mice were studied exclusively because skin thickness and collagen content are more uniform in male than in female mice.¹⁷

The dose and route of KW6002 administration were chosen based on previously published data on the pharmacokinetic properties of this compound.^{18–20} The dosing regimen was chosen for achieving drug concentrations that were sufficient for antagonizing the A_{2a}R but that still maintain specificity of A_{2a}R in mice. KW6002 25 mg/kg i.p.

administered in C57BL/6J mice reaches a maximum peak of serum concentration of 1030 ng/mL at 1.5 hours; the half-life of KW6002 is 11.6 hours. KW6002 is a selective A_{2a}R antagonist in mice, with a binding affinity of 1.87 nmol/L for A_{2a}R compared with 105.02 nmol/L for A₁R.²⁰ KW6002 has a binding affinity of 151.8 nmol/L for A_{2a}R compared with 11,169 nmol/L for A₁R, 2701 nmol/L for A_{2b}R, and 1939 nmol/L for A₃R when assessed in human receptors overexpressed in CHO and HEK293 cells.¹⁸ KW6002 administration at 10 mg/kg would result in an effective concentration of approximately 1 nmol/L, a concentration highly specific for A_{2a}R.²⁰

Mice were randomized to one of three treatment groups, which included negative controls (PBS), bleomycin plus vehicle (BLM; positive controls), and bleomycin plus KW6002 (BLM + KW6002). On day 24, the mice were sacrificed and the skinfold thickness was measured on the treated skin using skin calipers. The skin was shaved, excised, bisected, and fixed in 4% paraformaldehyde for routine histologic processing, or homogenized for the hydroxyproline assay. The number of mice per group was chosen based on the following power analysis: For >90% power for detecting a 70% reduction in the change in skin scores and numbers of cells per high-power field using an analysis of variance with repeated measures, with an α error probability of 0.05 and three groups, a minimum of five animals was needed for each condition. All protocols were approved by the New York University School of Medicine Institutional Animal Care and Use Committee.

Morphometric Dermal Measurements

Skin thickness was measured using a skin caliper on 12-mm punch biopsy samples obtained from the upper back, away from previous sites of injection. Skinfold (pinch) thickness was measured using a skin caliper on the same area over the middle to upper back of the mice. Breaking strength of the skin was measured using a forceps clamped to one side of the 12-mm skin biopsy sample and a tensiometer (Mark-10 Series EG Digital Force Gauge; Mark-10 Corp., Copiague, NY) coupled to another forceps clamped to the furthestmost extreme of the biopsy sample. The point of maximal stress before tearing of the sample was recorded. All measurements were undertaken in a blinded manner (J.Z.).⁶

Dermal Hydroxyproline Assay

Skin tissue specimens were hydrolyzed in 12N HCl at 120°C for 24 hours. After evaporation, 200 μ L of dextran-charcoal solution (1% charcoal and 0.05% dextran 500T) was mixed with 800 μ L of water and centrifuged at 400 $\times$ g for 10 minutes. A volume of 100 mL of a 1:2 dilution of the supernatant was mixed with 250 μ L of chloramine solution (1.3% chloramine-T, 10% propanol, and 80% citrate-acetate buffer) over 20 minutes at room temperature, followed by

the addition of 250 μ L of Ehrlich solution and incubation at 60°C for 20 minutes. Absorbance was measured at 550 nm. Standard curves (0 to 1 mg/mL) were generated for each experiment using reagent hydroxyproline as a standard. Results were expressed as micrograms of hydroxyproline per milligram of tissue.⁶

Histologic Examination, Immunofluorescence, and Image Analysis

Paraffin-embedded sections of skin were cut into serial sections of 5- μ m thickness and stained with hematoxylin and eosin, picosirius red (method of Puchtler).²¹

Immunofluorescence was performed on slides of paraffin-embedded skin. After deparaffination and rehydration of tissue sections, antigen retrieval was performed for 20 minutes at 98°C with 10 mmol/L citrate buffer pH 6.0. To reduce the staining background of immunofluorescence, the slides were incubated for 30 minutes with 2 mmol/L glycine. To block nonspecific binding, the slides were incubated for 1 hour with 5% fetal bovine serum in PBS—bovine serum albumin 3% Triton X-100 1%. Primary antibodies were incubated for 1 hour at room temperature. To identify nuclear accumulation of β -catenin in fibroblasts, skin sections were double-stained for GFP (1:200) or p-Ser552 β -catenin (1:200) or de-phospho β -catenin (1:200) and the fibroblast marker vimentin (mouse monoclonal antibody to vimentin, 1:800; rabbit monoclonal antibody to vimentin, 1:200). To identify myofibroblasts, skin sections were stained for α -SMA (1:200). Concentration-matched and species-specific immunoglobulins (Vector Laboratories, Burlingame, CA) were used as control antibodies. Secondary antibodies were labeled with anti-rabbit IgG—fluorescein isothiocyanate (green) and/or anti-mouse IgG—Alexa Fluor 555 (red) for 1 hour at room temperature. Nuclei were counterstained with DAPI mounting medium.

For hematoxylin and eosin—stained tissue, images were acquired using an SCN400 Digital Slide Scanner (Leica Microsystems, Wetzlar, Germany). For picosirius red, polarized images were collected with a DS-Fi1 camera (Nikon, Tokyo, Japan), and images were quantified with SigmaScan Pro software version 5 (Systat, Chicago, IL) in a blinded manner (J.Z.). The same yellow and red (hue 0, saturation 84, to hue 50, saturation 100) or green (hue 11, saturation 1, to hue 80, saturation 25) color threshold was applied to fields at 100-fold magnification for all of the images, and the area of each color was then calculated and normalized to PBS control as described.⁸ For immunofluorescence, images were collected with an Eclipse NI-U fluorescence upright microscope (Nikon). For the measurement of fluorescence intensity, all images were captured at the same exposure times, contrast settings, and intensity. For the quantification of fibroblasts with nuclear translocation of *Tcf/Lef:H2B-GFP* reporter or de-phospho β -catenin or p-Ser552 β -catenin, the percentage of nuclei stained for *Tcf/Lef:H2B-GFP* reporter or p-Ser552

β -catenin or de-phospho β -catenin, DAPI, and vimentin in the total number of single, spindle-shaped, DAPI-, and vimentin-positive fibroblastic cells was calculated in six randomly chosen high-power fields at 400-fold magnification in a blinded manner (J.Z.) as described.¹¹ For the quantification of myofibroblasts in the dermis, single, spindle-shaped cells positive for α -SMA were counted in six randomly chosen high-power fields at 200-fold magnification in a blinded manner (J.Z.) as described.⁸

Statistical Analysis

Results are represented as means $\pm$ SEM of separate determinations. Data were analyzed by one- or two-way analysis of variance, as appropriate, followed by Bonferroni post hoc test if F achieved $P < 0.05$ and there was no significant variance in homogeneity. All statistical analyses were performed with Prism software version 6.0 (GraphPad

Software, La Jolla, CA). The α nominal level was set at 0.05 in all cases. A P value of <0.05 was considered significant. All data and statistical analysis complied with the recommendations on experimental design and analysis set forth by *The American Journal of Pathology*.

Results

$A_{2a}R$ Pharmacologic Blockade Prevents Bleomycin-Induced Dermal Fibrosis

Our previous studies demonstrated that $A_{2a}R$ knockout and $A_{2a}R$ pharmacologic blockade (ZM241385) in wild-type mice prevent bleomycin-induced dermal fibrosis⁶ and radiation-induced dermal injury.⁸ Consistent with previous results, Wnt-signaling reporter mice treated with the $A_{2a}R$ antagonist (KW6002) were also protected from bleomycin-induced dermal fibrosis. We found that KW6002-treated

Figure 2 Adenosine A_{2a} receptor antagonist prevents myofibroblast accumulation in bleomycin (BLM)-induced dermal fibrosis. **A:** Anti- α -smooth muscle actin (α -SMA) immunofluorescence was performed on skin histologic sections from three groups. Nuclei are shown in blue (DAPI) and α -SMA in red. **B:** Numbers of α -SMA⁺ cells in skin sections were counted. Data are expressed as means $\pm$ SEM. $n = 5$ mice per group. * $P < 0.05$, ** $P < 0.01$. Original magnification: $\times 20$. HF, hair follicle; PBS, phosphate buffered saline.

Tcf/Lef:H2B-GFP mice displayed significantly reduced skin thickness (2.0 ± 0.3 mm versus 3.9 ± 0.3 mm, $P < 0.01$, $n = 5$) (Figure 1A) and skinfold thickness (5.5 ± 0.2 mm versus 9.6 ± 1.5 mm, $P < 0.05$, $n = 5$) (Figure 1B) compared with that in vehicle-treated mice after bleomycin challenge. These results were consistent with the higher dermal tensile strength, measured as breaking tension (810.5 ± 49.9 g versus 588.8 ± 44.7 g, $P < 0.05$, $n = 5$) (Figure 1C), and also higher dermal hydroxyproline content (40.3 ± 4.3 μ g/mg versus 25.0 ± 3.3 μ g/mg, $P < 0.05$, $n = 5$) (Figure 1D) in the vehicle-treated mice compared with KW6002-treated mice when challenged with bleomycin. Similarly, there was excessive accumulation of collagen and other extracellular matrix components, as seen in the hematoxylin and eosin-stained skin sections (Figure 1E), which was attenuated with KW6002 treatment. We further analyzed the collagen content of skin sections by histochemistry staining with picrosirius red, which, under polarizing microscopy, imparts intense yellow to red birefringence to thicker, more densely packed fibrils and weaker greenish birefringence to more loosely packed, thin fibrils, which may be immature collagen fibers.^{22,23} There was a marked increase in greenish birefringence ($492\% \pm 195\%$ versus $89\% \pm 27\%$ PBS, $P < 0.05$, $n = 5$) and yellow to red birefringence ($175\% \pm 5\%$ versus $132\% \pm 12\%$ PBS, $P < 0.05$, $n = 5$) in bleomycin-induced dermal fibrosis, which were both significantly decreased with KW6002 treatment (to $89\% \pm 27\%$ and $132\% \pm 12\%$ PBS, respectively, in treated mice, both $P < 0.05$) (Figure 1E). These results confirm that pharmacologic blockade of A_{2a}R attenuates the development of bleomycin-induced dermal fibrosis in the transgenic mice used in this murine model of scleroderma.

A_{2a}R Pharmacologic Blockade Prevents Myofibroblast Accumulation in Bleomycin-Induced Dermal Fibrosis

It is well documented that fibroblast/myofibroblast transition plays a pivotal role in the accumulation of excessive collagen and the contraction of the extracellular matrix associated with fibrosis.²⁴ During wound healing and progressive fibrotic diseases, fibroblasts transform into myofibroblasts, acquiring smooth muscle features, most notably the expression of α -SMA, the most widely used myofibroblast marker, and synthesis of mesenchymal cell-related matrix proteins.^{25,26} We observed an increase in the α -SMA positive myofibroblast population in bleomycin-induced dermal fibrosis, which was decreased with KW6002 treatment (13.6 ± 1.7 versus 7.2 ± 0.9 α -SMA⁺ cells/ $\times 20$ field, $P < 0.05$, $n = 5$) (Figure 2). These results indicate that A_{2a}R pharmacologic blockade partially prevents myofibroblast accumulation in bleomycin-induced dermal fibrosis, a finding that is in agreement with previously published results obtained from studies of radiation-induced dermal injury and scarring, and thioacetamide- and CCl₄-induced and ethanol-exacerbated liver fibrosis.^{8,20,22,27,28}

Pharmacologic Blockade of A_{2a}R Inhibits the Activation of β -Catenin in Bleomycin-Induced Dermal Fibrosis

To better detect Wnt/ β -catenin signaling activity in skin *in vivo*, we used *Tcf/Lef:H2B-GFP* mice, in which six copies of a Tcf/Lef response element and a heat shock protein 68 minimal promoter drive the expression of a fluorescent fusion protein comprising human histone H2B to GFP.^{16,29} Sensitivity of the *Tcf/Lef:H2B-GFP* reporter is supported by its

Figure 3 Adenosine A_{2a} receptor antagonist prevents nuclear translocation of *Tcf/Lef:H2B-GFP* reporter in bleomycin (BLM)-induced dermal fibrosis. **A:** Immunofluorescence images show staining against nuclei (DAPI, blue), Green fluorescent protein (GFP) (red), and vimentin (green) as well as overlay of these three stains in skin sections of three groups. **B:** The percentage of nuclei stained for GFP, DAPI, and vimentin in the total number of single, spindle-shaped, DAPI- and vimentin-positive fibroblasts were calculated in skin sections. Data are expressed as means $\pm$ SEM. $n = 5$ mice per group. *** $P < 0.001$, **** $P < 0.0001$. Original magnification: $\times 20$ (**A**, top rows); $40\times$ (**A**, bottom rows). G, gland; HF, hair follicle; HS, hair shaft; PBS, phosphate buffered saline.

activity at the sites of Wnt/ β -catenin signaling, confirmed through genetic analysis.¹⁶ The number and intensity of reporter gene expression were increased, indicating enhanced Wnt/ β -catenin signaling activity—not only in the fibroblasts of the dermis but also in the epidermis and eccrine glands of the dermis after bleomycin challenge—which was diminished with KW6002 treatment (Figure 3A). We further investigated the effect of the A_{2a} R antagonist KW6002 on nuclear translocation of β -catenin. Indeed, KW6002 treatment diminished the number of fibroblasts with nuclear translocation of *Tcf/Lef:H2B-GFP* reporter in bleomycin-challenged mice by 40% ($44\% \pm 6\%$ versus $74\% \pm 39\%$ of vimentin⁺ fibroblasts with GFP⁺ nuclei, $P < 0.001$, $n = 5$) (Figure 3B). Moreover, it was found that bleomycin induced nuclear translocation of *Tcf/Lef:H2B-GFP* reporter in epidermal keratinocytes and eccrine glands of the dermis, which was prevented with KW6002 treatment. Taken together, A_{2a} R pharmacologic blockade effectively inhibited the activation of β -catenin *in vivo*, which, at least in part, likely contributes to preventing bleomycin-induced dermal fibrosis in this murine model of scleroderma.

A_{2a} R Pharmacologic Blockade Inhibits Both Canonical and Akt Activation of β -Catenin in Bleomycin-Induced Dermal Fibrosis

Because it was found that A_{2a} R stimulation increases cellular β -catenin levels via both canonical activation and AKT activation *in vitro*,¹⁵ the expression of de-phospho β -catenin and p-Ser552 β -catenin after bleomycin challenge *in vivo* were also examined. In agreement with previous findings,¹⁵ the number and intensity of the expression of de-phospho β -catenin (Figure 4A and Supplemental Figure S1) and p-Ser552 (Figure 5A and Supplemental Figure S2) were increased in bleomycin-induced dermal fibrosis; these effects were diminished with KW6002 treatment. Moreover, fibroblasts with nuclear translocation of de-phospho β -catenin and p-Ser552 β -catenin were reduced by 55% (fibroblasts with de-phospho β -catenin—positive nuclei: BLM, $72\% \pm 5\%$; BLM + KW6002, $32\% \pm 3\%$; $P < 0.0001$, $n = 5$) (Figure 4B) and by 50% (fibroblasts with p-Ser552 β -catenin—positive nuclei: BLM, $76\% \pm 5\%$; BLM + KW6002, $38\% \pm 4\%$; $P < 0.001$, $n = 5$)

Figure 4 Adenosine A_{2a} receptor antagonist prevents nuclear translocation of dephosphorylated (dephospho) β -catenin in bleomycin (BLM)-induced dermal fibrosis. **A:** Immunofluorescence images show staining against nuclei (DAPI, blue), de-phospho β -catenin (green), and vimentin (red) as well as overlay of these three stains in skin sections from three groups. **B:** The percentages of nuclei stained for de-phospho β -catenin, DAPI, and vimentin in the total numbers of single, spindle-shaped, DAPI-, and vimentin-positive fibroblasts were calculated. Data are expressed as means $\pm$ SEM. *n* = 5 mice per group. *****P* < 0.0001. Original magnification, $\times 40$. HS, hair shaft; PBS, phosphate buffered saline.

(Figure 5B), respectively. Taken together, these results indicate that A_{2a}R pharmacologic blockade inhibits canonical and Akt activation of β -catenin *in vivo*, which, at least in part, contributes to the prevention of bleomycin-induced dermal fibrosis in this murine model of scleroderma.

Discussion

Previous studies have shown that treatment with an A_{2a}R antagonist (ZM241385) suppressed bleomycin-induced dermal fibrosis in wild-type C57Bl/6 mice.⁶ Here, we confirmed this finding using a different A_{2a}R antagonist, KW6002, in a different transgenic mouse strain, *Tcf/Lef:H2B-GFP* reporter mice, and found that KW6002 significantly reduced the severity of bleomycin-induced dermal fibrosis, as assessed by both histologic and immunohistochemistry staining. Additionally, there was increased expression of *Tcf/Lef:H2B-GFP* reporter in fibroblasts in bleomycin-induced dermal fibrosis, which was diminished with KW6002. Moreover, A_{2a}R blockade significantly inhibited nuclear translocation of *Tcf/Lef:H2B-GFP* reporter, as well as de-phospho and p-Ser552 β -catenin, in dermal fibroblasts. These findings confirm that there is crosstalk between A_{2a}R and Wnt/ β -catenin signaling *in vivo* in this murine model of bleomycin-induced dermal fibrosis.

Activation of the WNT/ β -catenin pathway is often a general feature of fibrotic diseases. Tissue samples from humans with fibrotic diseases, such as systemic sclerosis, idiopathic pulmonary fibrosis, and liver cirrhosis, showed enhanced expression of WNT proteins and decreased expression of Dickkopf-1, a secreted antagonist of WNT/ β -catenin signaling.³⁰ Nuclear accumulation of β -catenin in dermal fibroblasts was also observed in scleroderma patients and in animal models of scleroderma, including bleomycin-induced dermal fibrosis and tight skin-1 mice.^{11,30} Our findings in *Tcf/Lef:H2B-GFP* reporter mice in this model of bleomycin-induced dermal fibrosis are in accord with those from previous studies.^{11,30}

Crosstalk between WNT/ β -catenin signaling and the key mediator of fibrosis, transforming growth factor (TGF)- β , has been well documented.^{30,31} WNT/ β -catenin signaling has been shown to up-regulate the expression of TGF- β ₁, and TGF- β ₁ promotes β -catenin activation.^{30,31} Mice lacking Smad3, a downstream target of Tgf- β 1 signaling, demonstrate reduced β -catenin activation after wounding, and *Catnb* knockout fibroblasts have reduced Tgf- β —induced proliferation.³¹

Adenosine is released in response to tissue damage and can trigger a fibrotic cascade.¹ In particular, in previous studies, adenosine, released in response to bleomycin-induced generation of oxygen radicals in the skin, promoted dermal fibrosis via occupancy of A_{2a}R.^{7,9} A_{2a}R activation increases collagen synthesis in dermal fibroblasts

Figure 5 Adenosine A_{2a} receptor antagonist prevents nuclear translocation of phosphorylated (p)-Ser552 β -catenin in bleomycin (BLM)-induced dermal fibrosis. **A:** Immunofluorescence images show staining against nuclei (DAPI, blue), p-Ser552 β -catenin (green), and vimentin (red) as well as overlay of these three stains in skin sections from three groups. **B:** The percentages of nuclei stained for p-Ser552 β -catenin, DAPI, and vimentin in the total numbers of single, spindle-shaped, DAPI-, and vimentin-positive fibroblasts were calculated. Data are expressed as means $\pm$ SEM. $n = 5$ mice per group. ** $P < 0.01$, *** $P < 0.001$. Original magnification, $\times 40$. G, gland; HF, hair follicle; PBS, phosphate buffered saline.

via a pathway involving cAMP and AKT, which is independent of SMAD2/3.³² AKT, an activated downstream target of epidermal growth factor receptor signaling, can directly phosphorylate β -catenin at Ser552 *in vitro* and *in vivo*.³³ Activated AKT stabilizes β -catenin through inhibition of glycogen synthase kinase 3 β and/or directly phosphorylates β -catenin, resulting in its accumulation in the nucleus, which in turn stimulates β -catenin–TCF/LEF transcriptional activity.³³ Consistent with these observations, our recent data have demonstrated that $A_{2a}R$ activation increases cellular β -catenin levels in normal human dermal fibroblasts via both canonical activation and AKT activation. *In vivo*, we found that the $A_{2a}R$ antagonist KW6002 inhibited nuclear translocation of β -catenin, including glycogen synthase kinase 3 β –mediated dephospho β -catenin and Akt-activated p-Ser552 β -catenin, contributing to the prevention of bleomycin-induced dermal fibrosis.

Of note, we observed enhanced Wnt/ β -catenin signaling activity and nuclear translocation of β -catenin not only in the fibroblasts of the dermis but also in the keratinocytes and eccrine glands of the dermis after bleomycin challenge. β -Catenin has a dual role in cells: In addition to the WNT-signaling pathway, β -catenin also acts as a structural protein in connection with E-cadherin to form cellular adherens junctions, and participates in the process of epithelial–mesenchymal transition (EMT) in fibrosis.³⁴

During fibrogenic EMT, epithelial cells lose their polarity and cell–cell contact by down-regulating the expression of E-cadherin and releasing β -catenin from contacts, change their morphology to highly motile fibroblastoid mesenchymal cells, and express vimentin or α -SMA. The process of EMT is frequently accompanied by activation of the β -catenin signaling cascade. Activated β -catenin translocates from cytoplasm to nucleus and induces the transcription of EMT genes, such as those encoding vimentin, α -SMA, snail, slug, and twist, which in turn promotes the onset and progression of EMT.^{35,36} Thus, a significant increment of nuclear β -catenin in epidermis and epithelial appendages after bleomycin challenge hints at the existence of EMT in bleomycin-induced dermal fibrosis and likely reinforces Wnt/ β -catenin signaling due to the promotion of EMT.³⁷ We speculate that $A_{2a}R$ blockade may inhibit Wnt/ β -catenin signaling activity and α -SMA accumulation in fibroblasts in bleomycin-induced dermal fibrosis, in part by turning off the EMT process.

In the present study, we also observed that $A_{2a}R$ blockade prevented fibroblast/myofibroblast transition, as reflected by α -SMA accumulation, *in vivo*. Nonetheless, we failed to detect nuclear translocation of β -catenin in α -SMA⁺ myofibroblasts (data not shown). Several studies have addressed the relationship between α -SMA and β -catenin translocation, but the results to date have been controversial. Thus, TGF- β_1 induces α -SMA expression via glycogen

synthase kinase β inhibition and stimulates nuclear β -catenin translocation in human lung fibroblasts,³⁷ but in dermal fibroblasts, β -catenin signaling negatively regulates TGF- β ₁–induced myofibroblast transition.³⁸ Yet another study reported increased expression of stabilized β -catenin in skin fibroblasts, but this finding was not correlated with the presence of α -SMA⁺ myofibroblasts in stabilized β -catenin–induced skin fibrosis.³⁹ Our data also suggest that myofibroblast transformation may be independent of WNT signaling and β -catenin activation, although an A_{2a}R antagonist inhibits both WNT/ β -catenin signaling activity and α -SMA accumulation in bleomycin-induced dermal fibrosis. Because the α -SMA promoter does not contain TCF/LEF binding motifs, we also cannot rule out the possibility that the induction of α -SMA is secondary to the expression of other β -catenin–dependent genes.³⁷

A_{2a}R and A_{2b}R, the adenosine receptor subtypes most relevant to fibrosis, both signal via a Gs-mediated increase in cAMP, leading to the activation of downstream targets such as protein kinase A and exchange protein directly activated by cAMP 1/2. In human dermal fibroblasts and human hepatic stellate cells, cAMP-dependent signaling mediates the increase in collagen production as inhibition or knockdown of signaling proteins downstream of cAMP suppresses the A_{2a}R-mediated increase in collagen production.^{1,6,15,32,40} In contrast, A_{2b}R stimulation also increases cAMP levels, but stimulation of this receptor inhibits collagen production in cardiac fibroblasts.^{41–43} It is possible that downstream signaling proteins involved in collagen production differ between cardiac and dermal or hepatic fibroblasts. However, it is more likely that cells stimulated by these two receptor subtypes differ with regard to the levels or locations of increases in cAMP. But the regulatory actions of the receptors likely involve multiple interconnected pathways; one aspect of this regulation is the opposing effect of the different levels of cAMP generated. Perez-Aso et al^{32,40} reported that forskolin, an agent that directly stimulates adenylate cyclase and which is often used to increase cAMP level in studies of signaling mechanisms, stimulates much more intracellular cAMP accumulation than does A_{2a}R and also results in inhibition of collagen production by fibroblasts. Although not tested directly, these findings are consistent with the hypothesis that quantitative or geographic differences in intracellular cAMP generation resulting from A_{2a}R and A_{2b}R stimulation dictate different downstream signaling effects and the differing effects on collagen production.

Conclusion

We report here that the A_{2a}R antagonist KW6002 inhibits the Wnt/ β -catenin signaling pathway *in vivo*, which contributes to preventing bleomycin-induced dermal fibrosis. The use of KW6002, which improves motor function in animal models of Parkinson disease and in patients with

Parkinson disease, has been approved in Japan as the first A_{2a}R selective antagonist for clinical application.⁴⁴ Our results provide evidence supporting off-label use of this drug and represents a potential novel therapeutic modality for the treatment and prevention of dermal fibrosis.

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Supplemental Data

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References

1. Shaikh G, Cronstein B: Signaling pathways involving adenosine A_{2A} and A_{2B} receptors in wound healing and fibrosis. *Purinergic Signal* 2016, 12:191–197
2. Wynn TA: Cellular and molecular mechanisms of fibrosis. *J Pathol* 2008, 214:199–210
3. Sakkas LI, Simopoulou T, Katsiari C, Bogdanos D, Chikanza IC: Early systemic sclerosis-opportunities for treatment. *Clin Rheumatol* 2015, 34:1327–1331
4. Eltzschig HK, Eckle T: Ischemia and reperfusion—from mechanism to translation. *Nat Med* 2011, 17:1391–1401
5. Borea PA, Gessi S, Merighi S, Varani K: Adenosine as a multi-signalling guardian angel in human diseases: when, where and how does it exert its protective effects? *Trends Pharmacol Sci* 2016, 37: 419–434
6. Chan ES, Fernandez P, Merchant AA, Montesinos MC, Trzaska S, Desai A, Tung CF, Khoa DN, Pillinger MH, Reiss AB, Tomic-Canic M, Chen JF, Schwarzschild MA, Cronstein BN: Adenosine A_{2A} receptors in diffuse dermal fibrosis: pathogenic role in human dermal fibroblasts and in a murine model of scleroderma. *Arthritis Rheum* 2006, 54:2632–2642
7. Fernandez P, Perez-Aso M, Smith G, Wilder T, Trzaska S, Chiriboga L, Franks A Jr, Robson SC, Cronstein BN, Chan ES: Extracellular generation of adenosine by the ectonucleotidases CD39 and CD73 promotes dermal fibrosis. *Am J Pathol* 2013, 183: 1740–1746
8. Perez-Aso M, Mediero A, Low YC, Levine J, Cronstein BN: Adenosine A_{2A} receptor plays an important role in radiation-induced dermal injury. *FASEB J* 2016, 30:457–465
9. Fernandez P, Trzaska S, Wilder T, Chiriboga L, Blackburn MR, Cronstein BN, Chan ES: Pharmacological blockade of A_{2A} receptors prevents dermal fibrosis in a model of elevated tissue adenosine. *Am J Pathol* 2008, 172:1675–1682
10. Clevers H: Wnt/ β -catenin signaling in development and disease. *Cell* 2006, 127:469–480
11. Beyer C, Schramm A, Akhmetshina A, Dees C, Kireva T, Gelse K, Sonnylal S, de Crombrughe B, Taketo MM, Distler O, Schett G, Distler JH: β -catenin is a central mediator of pro-fibrotic Wnt signaling in systemic sclerosis. *Ann Rheum Dis* 2012, 71:761–767
12. Cheon SS, Wei Q, Gurung A, Youn A, Bright T, Poon R, Whetstone H, Guha A, Alman BA: β -catenin regulates wound size

- and mediates the effect of TGF-beta in cutaneous healing. *FASEB J* 2006, 20:692–701
13. Guo Y, Xiao L, Sun L, Liu F: Wnt/beta-catenin signaling: a promising new target for fibrosis diseases. *Physiol Res* 2012, 61:337–346
 14. Verheyen EM, Gottardi CJ: Regulation of Wnt/beta-catenin signaling by protein kinases. *Dev Dyn* 2010, 239:34–44
 15. Shaikh G, Zhang J, Perez-Aso M, Mediero A, Cronstein B: Adenosine A2A receptor promotes collagen type III synthesis via beta-catenin activation in human dermal fibroblasts. *Br J Pharmacol* 2016, 173:3279–3291
 16. Ferrer-Vaquer A, Piliszek A, Tian G, Aho RJ, Dufort D, Hadjantonakis AK: A sensitive and bright single-cell resolution live imaging reporter of Wnt/ss-catenin signaling in the mouse. *BMC Dev Biol* 2010, 10:121
 17. Markova MS, Zeskand J, McEntee B, Rothstein J, Jimenez SA, Siracusa LD: A role for the androgen receptor in collagen content of the skin. *J Invest Dermatol* 2004, 123:1052–1056
 18. Yang M, Soohoo D, Soelaiman S, Kalla R, Zablocki J, Chu N, Leung K, Yao L, Diamond I, Belardinelli L, Shryock JC: Characterization of the potency, selectivity, and pharmacokinetic profile for six adenosine A2A receptor antagonists. *Naunyn Schmiedebergs Arch Pharmacol* 2007, 375:133–144
 19. Borgkvist A, Marcellino D, Fuxe K, Greengard P, Fisone G: Regulation of DARPP-32 phosphorylation by Delta9-tetrahydrocannabinol. *Neuropharmacology* 2008, 54:31–35
 20. Chiang DJ, Roychowdhury S, Bush K, McMullen MR, Pisano S, Niese K, Olman MA, Pritchard MT, Nagy LE: Adenosine 2A receptor antagonist prevented and reversed liver fibrosis in a mouse model of ethanol-exacerbated liver fibrosis. *PLoS One* 2013, 8:e69114
 21. Heap BJ, Kiernan JA: Mast cells and gastric secretion in the rat. *Q J Exp Physiol Cogn Med Sci* 1975, 60:307–313
 22. Perez-Aso M, Chiriboga L, Cronstein BN: Pharmacological blockade of adenosine A2A receptors diminishes scarring. *FASEB J* 2012, 26:4254–4263
 23. Whittaker P, Kloner RA, Boughner DR, Pickering JG: Quantitative assessment of myocardial collagen with picrosirius red staining and circularly polarized light. *Basic Res Cardiol* 1994, 89:397–410
 24. McAnulty RJ: Fibroblasts and myofibroblasts: their source, function and role in disease. *Int J Biochem Cell Biol* 2007, 39:666–671
 25. Hinz B: Formation and function of the myofibroblast during tissue repair. *J Invest Dermatol* 2007, 127:526–537
 26. Barnes JL, Gorin Y: Myofibroblast differentiation during fibrosis: role of NAD(P)H oxidases. *Kidney Int* 2011, 79:944–956
 27. Chan ES, Montesinos MC, Fernandez P, Desai A, Delano DL, Yee H, Reiss AB, Pillinger MH, Chen JF, Schwarzschild MA, Friedman SL, Cronstein BN: Adenosine A(2A) receptors play a role in the pathogenesis of hepatic cirrhosis. *Br J Pharmacol* 2006, 148:1144–1155
 28. Peng Z, Fernandez P, Wilder T, Yee H, Chiriboga L, Chan ES, Cronstein BN: Ecto-5'-nucleotidase (CD73) -mediated extracellular adenosine production plays a critical role in hepatic fibrosis. *FASEB J* 2008, 22:2263–2272
 29. Choi YS, Zhang Y, Xu M, Yang Y, Ito M, Peng T, Cui Z, Nagy A, Hadjantonakis AK, Lang RA, Cotsarelis G, Andl T, Morrissey EE, Millar SE: Distinct functions for Wnt/beta-catenin in hair follicle stem cell proliferation and survival and interfollicular epidermal homeostasis. *Cell Stem Cell* 2013, 13:720–733
 30. Akhmetshina A, Palumbo K, Dees C, Bergmann C, Venalis P, Zerr P, Horn A, Kireva T, Beyer C, Zwerina J, Schneider H, Sadowski A, Riener MO, MacDougald OA, Distler O, Schett G, Distler JH: Activation of canonical Wnt signalling is required for TGF-beta-mediated fibrosis. *Nat Commun* 2012, 3:735
 31. Zhou B, Liu Y, Kahn M, Ann DK, Han A, Wang H, Nguyen C, Flodby P, Zhong Q, Krishnaveni MS, Liebler JM, Minoo P, Crandall ED, Borok Z: Interactions between beta-catenin and transforming growth factor-beta signaling pathways mediate epithelial-mesenchymal transition and are dependent on the transcriptional co-activator cAMP-response element-binding protein (CREB)-binding protein (CBP). *J Biol Chem* 2012, 287:7026–7038
 32. Perez-Aso M, Fernandez P, Mediero A, Chan ES, Cronstein BN: Adenosine 2A receptor promotes collagen production by human fibroblasts via pathways involving cyclic AMP and AKT but independent of Smad2/3. *FASEB J* 2014, 28:802–812
 33. Fang D, Hawke D, Zheng Y, Xia Y, Meisenhelder J, Nika H, Mills GB, Kobayashi R, Hunter T, Lu Z: Phosphorylation of beta-catenin by AKT promotes beta-catenin transcriptional activity. *J Biol Chem* 2007, 282:11221–11229
 34. Kalluri R, Weinberg RA: The basics of epithelial-mesenchymal transition. *J Clin Invest* 2009, 119:1420–1428
 35. Bose SK, Meyer K, Di Bisceglie AM, Ray RB, Ray R: Hepatitis C virus induces epithelial-mesenchymal transition in primary human hepatocytes. *J Virol* 2012, 86:13621–13628
 36. Polimeni M, Gulino GR, Gazzano E, Kopecka J, Marucco A, Fenoglio I, Cesano F, Campagnolo L, Magrini A, Pietroiusti A, Ghigo D, Aldieri E: Multi-walled carbon nanotubes directly induce epithelial-mesenchymal transition in human bronchial epithelial cells via the TGF-beta-mediated Akt/GSK-3beta/SNAIL-1 signalling pathway. *Part Fibre Toxicol* 2016, 13:27
 37. Caraci F, Gili E, Calafiore M, Failla M, La Rosa C, Crimi N, Sortino MA, Nicoletti F, Copani A, Vancheri C: TGF-beta1 targets the GSK-3beta/beta-catenin pathway via ERK activation in the transition of human lung fibroblasts into myofibroblasts. *Pharmacol Res* 2008, 57:274–282
 38. Liu J, Wang Y, Pan Q, Su Y, Zhang Z, Han J, Zhu X, Tang C, Hu D: Wnt/beta-catenin pathway forms a negative feedback loop during TGF-beta1 induced human normal skin fibroblast-to-myofibroblast transition. *J Dermatol Sci* 2012, 65:38–49
 39. Hamburg-Shields E, DiNucio GJ, Mullin NK, Lafyatis R, Atit RP: Sustained beta-catenin activity in dermal fibroblasts promotes fibrosis by up-regulating expression of extracellular matrix protein-coding genes. *J Pathol* 2015, 235:686–697
 40. Perez-Aso M, Mediero A, Cronstein BN: Adenosine A2A receptor (A2AR) is a fine-tune regulator of the collagen1:collagen3 balance. *Purinergic Signal* 2013, 9:573–583
 41. Insel PA, Murray F, Yokoyama U, Romano S, Yun H, Brown L, Snead A, Lu D, Aroonsakool N: cAMP and Epac in the regulation of tissue fibrosis. *Br J Pharmacol* 2012, 166:447–456
 42. Lu D, Aroonsakool N, Yokoyama U, Patel HH, Insel PA: Increase in cellular cyclic AMP concentrations reverses the profibrogenic phenotype of cardiac myofibroblasts: a novel therapeutic approach for cardiac fibrosis. *Mol Pharmacol* 2013, 84:787–793
 43. Lu D, Insel PA: Hydrolysis of extracellular ATP by ectonucleoside triphosphate diphosphohydrolase (ENTPD) establishes the set point for fibrotic activity of cardiac fibroblasts. *J Biol Chem* 2013, 288:19040–19049
 44. Stayte S, Vissel B: Advances in non-dopaminergic treatments for Parkinson's disease. *Front Neurosci* 2014, 8:113