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RESEARCH PAPER

Adenosine A_{2A} receptor promotes collagen type III synthesis via β-catenin activation in human dermal fibroblasts

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BACKGROUND AND PURPOSE

Adenosine A_{2A} receptor stimulation promotes the synthesis of collagen type I and type III (Col1 and Col3), mediators of fibrosis and scarring. The A_{2A} receptor modulates collagen balance via cAMP/PKA/p38-MAPK/Akt pathways. Wnt signalling is important in fibrosis and the cAMP and Wnt pathways converge. Because the A_{2A} receptor is Gs-linked and increases cAMP, we determined whether A_{2A} receptors and Wnt signalling interact.

EXPERIMENTAL APPROACH

Total β-catenin, de-phosphorylated β-catenin (canonical activation, de-phospho β-catenin) and phosphorylated β-catenin at Ser^{552} (non-canonical activation, p-Ser^{552} β-catenin) levels were determined in primary human dermal fibroblasts, cytosol and nucleus, by western blot analysis and fluorescence microscopy, before and after stimulation by A_{2A} receptor-selective agonist CGS21680, with/without A_{2A} receptor-selective antagonist (SCH56261) pretreatment. β-Catenin was knocked down by transfection with scrambled-siRNA or specific-siRNA, and Col1 and Col3 levels determined by western blots.

KEY RESULTS

CGS21680 stimulation rapidly (15 min) increased cellular β-catenin levels. Both de-phospho β-catenin and p-Ser^{552} β-catenin levels were also increased. CGS21680 stimulated the translocation of total de-phospho and p-Ser^{552} β-catenin to the nucleus. A_{2A} receptor-stimulation increased Col1 synthesis similarly in β-catenin knockeddown and scrambled cells. However, β-catenin knockdown abolished the increase in Col3 synthesis induced in A_{2A} receptor-stimulated fibroblasts.

CONCLUSIONS AND IMPLICATIONS

A_{2A} receptor stimulation promotes Col3 synthesis via the activation of canonical and non-canonical β-catenin, consistent with a role for A_{2A} receptors in dermal fibrosis and scarring.

Abbreviations

Col1, collagen type I; Col3, collagen type III; CBP, cofactor CREB-binding protein; CREB, cAMP response element binding protein; CTGF, connective tissue growth factor; NHDFs, normal human dermal fibroblasts

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Tables of Links

TARGETS	
GPCRs[a]	**Enzymes**[c]
A_{2A} receptor	Akt (PKB)
Other protein targets[b]	ERK1
CBP (CREB binding protein)	ERK2
	GSK3β
	p38 MAPK
	PKA
	Smad

LIGANDS
β-catenin
cAMP
CGS21680
CTGF
SCH58261
TGF-β1

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan *et al.*, 2016) and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 ([a,b,c]Alexander *et al.*, 2015a,b,c).

Introduction

Cutaneous wound healing is initially characterized by inflammation followed by the formation of new vessels and accumulation of matrix elements. Later, re-epithelialization, scar contraction and remodelling occur (Andrews *et al.*, 2016). However, the wound healing process can be diverted or become overly active leading to excessive matrix production. Dermal fibrosis, which is the pathological remodelling of skin characterized by the excessive accumulation of collagen and other matrix elements, underlies a wide range of clinically important diseases including hypertrophic scarring, keloid and morphea (Andrews *et al.*, 2016; Shaikh and Cronstein, 2016). Scleroderma and other diffuse fibrosing conditions, although uncommon, cause multiple morbidities in affected people, including disfigurement and a diminished range of movements as well as premature mortality as a result of renal and cardiac dysfunctions (Balbir-Gurman and Braun-Moscovici, 2012).

Adenosine is a purine nucleoside generated extracellularly from adenine nucleotides, such as ATP and ADP, and is released by cells as a result of direct stimulation, hypoxia, injury or metabolic stress (Eltzschig and Eckle, 2011; Borea *et al.*, 2016). Adenosine levels in the milieu of ischaemic or necrotic tissue can increase several orders of magnitude, from nanomolar to millimolar concentrations (Montesinos *et al.*, 2002). Adenosine activates adenylyl cyclase leading to an increase in intracellular cAMP by stimulation of a family of G_s-coupled protein receptors; A_{2A} and A_{2B} receptors being the principal ones involved with respect to fibrosis (Hasko *et al.*, 2008; Fredholm *et al.*, 2011). The increase in cAMP, in turn, activates PKA and other downstream targets that influence the expression of genes associated with wound healing and fibrosis (Hasko and Cronstein, 2004; Chan *et al.*, 2006; Mediero *et al.*, 2013; Borea *et al.*, 2016).

The overproduction of A_{2A} receptors, resulting from a deficiency in adenosine deaminase, leads to marked fibrosis (Fernandez *et al.*, 2008). Deletion or blockade of A_{2A} receptors has been shown to completely prevent hepatic and dermal fibrosis in murine models (Perez-Aso *et al.*, 2012). Furthermore, reducing the capacity to produce extracellular adenosine as a result of CD73 or CD39 deletion also markedly reduces fibrosis in murine models (Fernandez *et al.*, 2013). A_{2A} receptor activation has been shown to stimulate fibroblasts directly and indirectly to produce collagen type I and type III (Col1 and Col3) through different mechanisms (Chan *et al.*, 2013; Perez-Aso *et al.*, 2013). In addition, different levels of receptor engagement have been shown to promote different types of collagen synthesis (Chan *et al.*, 2013; Perez-Aso *et al.*, 2013). For example, at low levels of receptor stimulation, Col1 expression is promoted in a pathway involving Akt phosphorylation and activation. However, higher levels of receptor stimulation induce the expression of Col3 via p38 MAPK-dependent signalling (Perez-Aso *et al.*, 2013; 2014). The different levels of receptor stimulation required for production of these different collagen subtypes probably reflects, in part, the fact that Col3 expression is negatively regulated by ERK1 and ERK2 and higher levels of receptor activation are required to overcome this inhibition (Perez-Aso *et al.*, 2014). Thus, A_{2A} receptor-mediated collagen production contributes to normal wound healing and scar formation by multiple signalling pathways and also participates in the pathogenesis of fibrotic conditions such as scleroderma and cirrhosis (Fernandez *et al.*, 2008; Perez-Aso *et al.*, 2012).

The role of Wnt/β-catenin signalling in wound healing and fibrosis has also been extensively investigated (Cheon *et al.*, 2006; Poon *et al.*, 2009; Wei *et al.*, 2011). Canonical Wnts are lipoglycoproteins that are secreted and involved in embryological development, cellular fating and normal homeostasis by activating membrane-bound frizzled receptors (Wodarz and Nusse, 1998; Reya *et al.*, 2003; van Es *et al.*, 2005; Clevers, 2006). Wnt-mediated signalling results in the activation of a transcription complex that contains the cytosolic protein β-catenin and its DNA-binding partners, lymphocyte enhancer factor (LEF)/T-cell factor (TCF) (Clevers, 2006). Wnt binding to the frizzled receptor stimulates a rise in cytosolic β-catenin through inhibition of a multi-subunit protein complex known as the 'β-catenin destruction complex', which includes the key regulatory enzyme GSK3β. In the absence of Wnt binding to the frizzled receptor, GSK3β constitutively phosphorylates

β-catenin, leading to its ubiquitination and destruction by the proteasome (Clevers, 2006). By inhibiting this phosphorylation, β-catenin accumulates in the cytoplasm and translocates to the nucleus, where it can complex with the LEF/TCF family of proteins and localize to its gene promoters (Clevers, 2006). Other non-canonical Wnt signalling pathways have also been described (Komiya and Habas, 2008).

The activation of β-catenin-dependent Wnt signalling is one of the initial cellular responses to tissue injury. Wnt/β-catenin signalling in fibroblast cultures enhances the proliferation, migration and extracellular matrix production of these cells (Wei *et al.,* 2011). Furthermore, cutaneous wound healing studies in mice have demonstrated that β-catenin signalling is activated after injury, and a mutated form of β-catenin resistant to ubiquitin-mediated degradation leads to excessive collagen synthesis and hyperplastic scarring (Cheon *et al.,* 2002). Additionally, β-catenin induces the contraction of collagen lattices, an important step in the maturation of scars (Poon *et al.,* 2009). Crosstalk between Wnt/β-catenin and another important mediator of fibrosis, TGF-β 1, has also been demonstrated to occur (Zhou *et al.,* 2012). Wnt/β-catenin signalling has been shown to up-regulate the expression of TGF-β1, and TGF-β1 promotes β-catenin signalling (Cheon *et al.,* 2002; Zhou *et al.,* 2012). Mice lacking Smad3, a downstream target of TGF-β1 signalling, have been demonstrated to have lower levels of β-catenin activation after injury, and TGF-β1-induced proliferation of fibroblasts is reduced in β-catenin knockedout cells (Cheon *et al.,* 2002). Nevertheless, the relationship between β-catenin and TGF-β 1 signalling is complex, and there is also evidence of antagonism between the two pathways (Liang *et al.,* 2008).

Recent evidence indicates a convergence of cAMP, the main effector of A_{2A} receptor stimulation, and Wnt/ β-catenin signalling, which prompted us to analyse the crosstalk between these two signalling pathways (Hino *et al.,* 2005). It has also been shown that direct phosphorylation of β-catenin by Akt promotes β-catenin transcriptional activity, representing a possible A_{2A} receptor-mediated non-canonical pathway for its activation (Fang *et al.,* 2007). Additionally, although both TGF-β1 and adenosine promote fibrosis, they follow different signalling pathways wherein A_{2A} receptor stimulation promotes collagen production via cAMP and Akt but independently of Smad2/3 (Perez-Aso *et al.,* 2014). Thus, Wnt/β-catenin signalling might represent a final common pathway for these two important mediators of fibrosis, and the results of this study suggest that directly modifying these signalling events might represent a novel approach for diminishing scarring and fibrosis without affecting cutaneous wound healing.

Methods

Stimulation and preparation of cellular extracts and western blots

NHDF cells were cultured in DMEM containing 10% FBS and 1% penicillin/streptomycin (DMEM) and used for experiments from the first to fifth passages. β-catenin siRNA transfected NHDF cells (siRNA to β-catenin or scrambled siRNA as control) and NHDF cells were stimulated with the A_{2A} receptor-selective agonist CGS21680 at increasing doses ranging from 0.1 to 10 μM for the indicated time period (dose–response) or at an indicated dose for different time periods (time–course). When used, the A_{2A} receptor-selective antagonist SCH58261 (1 μM) was introduced 30 min before CGS21680. For all the experiments with antagonists, a dose–response curve from 0.1 to 10 μM of CGS21680 was run at the same time. After being stimulated, cells were washed with cold PBS and lysed in RIPA buffer containing protease inhibitor cocktail and phosphatase inhibitor cocktail to isolate total cell protein content. The extraction of cytoplasmic and nuclear fraction proteins was carried out using NE-PER nuclear and cytoplasmic extraction reagent kits. Protein concentration was quantified by BCA protein assay (Mediero *et al.,* 2014). Next, 4 μg of protein extract for β-catenin, de-phospho-β-catenin ($Ser^{33/37}$/Thr^{41}) and phospho-β-catenin (Ser552), 3 μg for Col1 and 10 μg for Col3 were separated by use of SDS-PAGE and transferred to nitrocellulose membranes for immunoblotting. After being blocked in Tris-buffered saline with 0.1% Tween 20 (TBST) plus 3% BSA (Sigma-Aldrich) for 1 h at room temperature, membranes were incubated overnight at 4°C with primary antibodies against Col1 (1:500), Col3 (1:500), β-catenin (1:1000), de-phospho-β-catenin ($Ser^{33/37}$/Thr^{41}) (1:1000), phospho-β-catenin (Ser^{552}) (1:1000), p84 (1:1000) and actin (1:1000). The membranes were then washed with TBST and incubated with goat anti-rabbit IRDye 800CW (1:5000) and goat anti-mouse IRDye 680RD (1:5000), or donkey anti-goat IRDye 800CW (1:5000) and donkey anti-mouse IRDye 680RD (1:5000) for 1 h at room temperature in the dark, and images were visualized with Li-Cor Odyssey equipment (Li-Cor Biosciences) where near-infrared fluorescent signals were detected, because each secondary antibody emits a signal at a different frequency. A specific nuclear signal was determined using mouse monoclonal anti-nuclear matrix protein p84. The intensities of the respective bands were quantified by densitometric analysis using the Image Studio 2.0.38 software (Li-Cor Biosciences) (Mediero *et al.,* 2014).

Band quantification was first normalized to actin or p84, and then, the percentage was calculated against the non-stimulated control blotted on the same membrane. Data obtained from densitometric analyses were normalized to account for unequal loading of samples across the lanes on a gel and for differences in transfer efficiency across a blot.

RNA interference (siRNA)

Double-stranded siRNAs against β-catenin (4390824 (s436)) and scrambled siRNAs (4390844) as a negative control were purchased from Ambion (Life Technologies, Grand Island, NY, USA). NHDF cells were transfected with siRNA (50 pmol for β-catenin knockdown with the negative control at the respective concentrations), using Lipofectamine RNAiMAX (Invitrogen, Life Technologies). After 24 h, CGS21680 (1 μM) was added for 24 h, and cellular extracts were prepared as described above.

Immunocytochemistry

To identify the activation and nuclear translocation of β-catenin, NHDF cells were plated on glass coverslips (Lab-Tek II chamber slide, 8-well; Thermo Fisher Scientific) in DMEM. When they reached 75% confluence, cells were fixed with cold 4% paraformaldehyde (Hatfield, PA, USA) in PBS for

15 min at room temperature. After being washed with PBS for 10 min twice and PBS containing BSA 2% for 10 min once, the cells were blocked with PBS BSA 2% Triton X-100 0.5% FBS 5% for 1 h at room temperature. Next, cells were incubated overnight at 4°C with primary antibodies against β-catenin (1:400), de-phospho-β-catenin ($Ser^{33/37}/Thr^{41}$) (1:400) and phospho-β-catenin (Ser^{552}) (1:400). Then, cells were washed with PBS containing BSA 2% and incubated with goat anti-rabbit IgG (whole molecule)-FITC for 1 h at room temperature in the dark. After being washed with PBS containing BSA 2% for 10 min twice and PBS for 10 min once, cells were counterstained with DAPI and each sample was examined using a fluorescence microscope (Nikon Eclipse NI-U fluorescence uplight microscope, Tokyo, Japan).

Statistical analysis

Results are presented as mean ± SEM. Data were analysed by one or two-way ANOVA followed by Bonferroni *post hoc* test if F achieved $P < 0.05$, and there was no significant variance in-homogeneity or by Student's t-test. All statistical analyses were performed with GRAPHPAD PRISM software v. 6.0 (Graphpad Software, La Jolla, CA, USA). The α nominal level was set at 0.05 in all cases. A P value of <0.05 was the considered significant. Randomization was not performed, as this experiment did not involve animals or human subjects. Additionally, explicit blinding was not performed; however, squares of identical areas were used to measure pixel density to eliminate bias across experimental results. The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2015).

Antibodies, reagents and cell line

Normal human dermal fibroblasts (NHDFs) were purchased from Lonza (Walkersville, MD, USA). DMEM (high glucose), FBS, penicillin/streptomycin, NE-PER nuclear and cytoplasmic extraction reagents kit, and bicinchoninic acid (BCA)

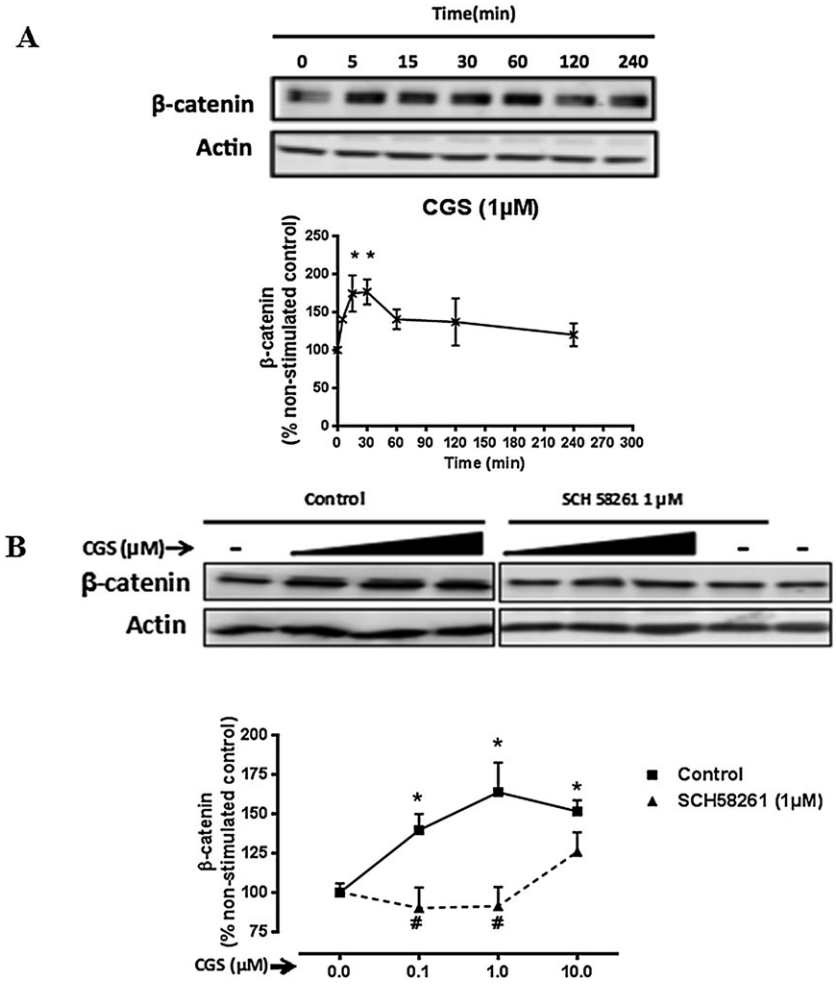

Figure 1

Activation of A_{2A} receptors rapidly increases cellular β-catenin levels. (A) NHDF cells were incubated with CGS21680 (1 μM) for periods of up to 4 h and collected at the indicated time-points. (B) NHDF cells were incubated with increasing concentrations of CGS21680 (0.1–10 μM) for 15 min, with or without SCH58261 (1 μM) pre-incubation. Cellular β-catenin levels were measured. Representative images of western blots for β-catenin and actin are shown. Data represent mean ± SEM of six independent experiments as determined by densitometry relative to actin. *$P < 0.05$ versus non-stimulated control; #$P < 0.05$, SCH58261 + CGS26180 versus CGS26180. CGS indicates CGS26180.

Protein Assay Reagent were purchased from Thermo Fisher Scientific (Grand Island, NY, USA). CGS21680 and SCH58261 were purchased from Tocris Bioscience (Ellisville, MO, USA). Rabbit monoclonal antibodies to β-catenin and de-phospho-β-catenin ($Ser^{33/37/}Thr^{41}$) and phospho-β-catenin (Ser^{552}) were purchased from Cell Signalling Technology (Danvers, MA, USA). Goat antibodies to Col1 and Col3 were purchased from SouthernBiotech (Birmingham, AL, USA). Mouse monoclonal anti-nuclear matrix protein p84 was purchased from Abcam (Cambridge, MA, USA). Mouse monoclonal antibody to actin (C-2) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Goat anti-rabbit IRDye 800CW, goat anti-mouse IRDye 680 RD, donkey anti-goat IRDye 800CW and donkey anti-mouse IRDye 680 RD were purchased from Li-Cor Biosciences (Lincoln, NE, USA). DAPI mounting medium, goat anti-rabbit IgG (whole molecule)-FITC, the RIPA buffer, protease inhibitor cocktail and phosphatase inhibitor cocktail were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Results

A_{2A} receptor activation rapidly increases cellular β-catenin levels

To explore the effects of A_{2A} receptor stimulation on β-catenin levels in dermal fibroblasts, we first determined the time–course of the effects of stimulation with the A_{2A} receptor-selective agonist, CGS21680, on β-catenin levels in NHDFs. We found that after stimulation with CGS21680 (1 μM), cellular β-catenin levels rapidly increased before achieving a peak at 15 min (174 ± 23% of non-stimulated control, $P < 0.05$, $n = 6$) and 30 min (176 ± 16% of non-stimulated control, $P < 0.05$, $n = 6$) (Figure 1A). The A_{2A} receptor-mediated increase in β-catenin levels in NHDFs was dose-dependent (Figure 1B). We observed that CGS21680 stimulation at concentrations ranging from nanomolar to micromolar increased cellular β-catenin levels, and pretreatment of NHDFs with the highly selective A_{2A} receptor antagonist SCH58261 (1 μM) completely reversed the effect of CGS21680 on β-catenin levels (Figure 1B). These results are consistent with the hypothesis that A_{2A} receptor activation prevents β-catenin degradation, thereby rapidly increasing total cellular β-catenin levels.

Activation of A_{2A} receptors increases cellular β-catenin levels by both canonical and Akt-mediated activation

To better define how A_{2A} receptor activation increases β-catenin expression in dermal fibroblasts, we investigated the levels of β-catenin after CGS21680 stimulation in NHDFs. Because previous studies have demonstrated that both canonical and non-canonical activation of β-catenin leads to an up-regulation of the expression of its gene, we examined the expression of both de-phosphorylated β-catenin (canonical β-catenin activation, de-phospho β-catenin) and phosphorylated β-catenin at Ser^{552} (non-canonical β-catenin activation, site of

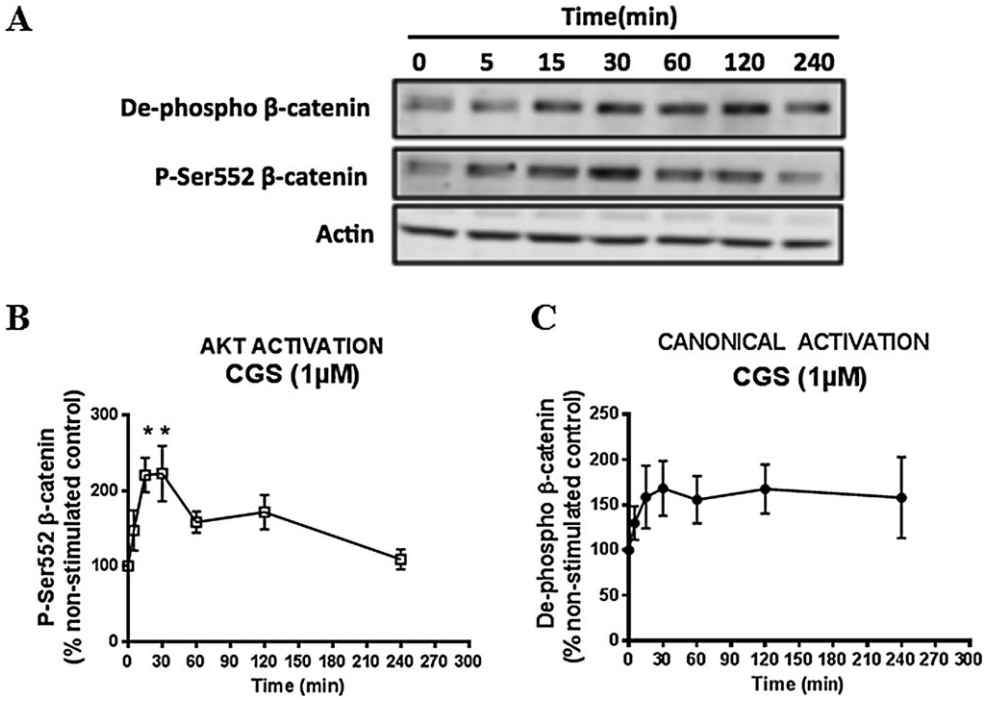

Figure 2

Activation of A_{2A} receptors increases cellular β-catenin levels via canonical activation and Akt activation. NHDF cells were incubated with CGS21680 (1 μM) for periods of up to 4 h and collected at the indicated time-points. (A) Representative images of western blots for de-phospho-β-catenin, p-Ser^{552}-β-catenin and actin are shown. Effects of CGS21680 stimulation on (B) p-Ser^{552}-β-catenin and (C) de-phospho-β-catenin were analysed. Data represent mean ± SEM of six independent experiments as determined by densitometry relative to actin. *$P < 0.05$ versus non-stimulated control. CGS indicates CGS26180.

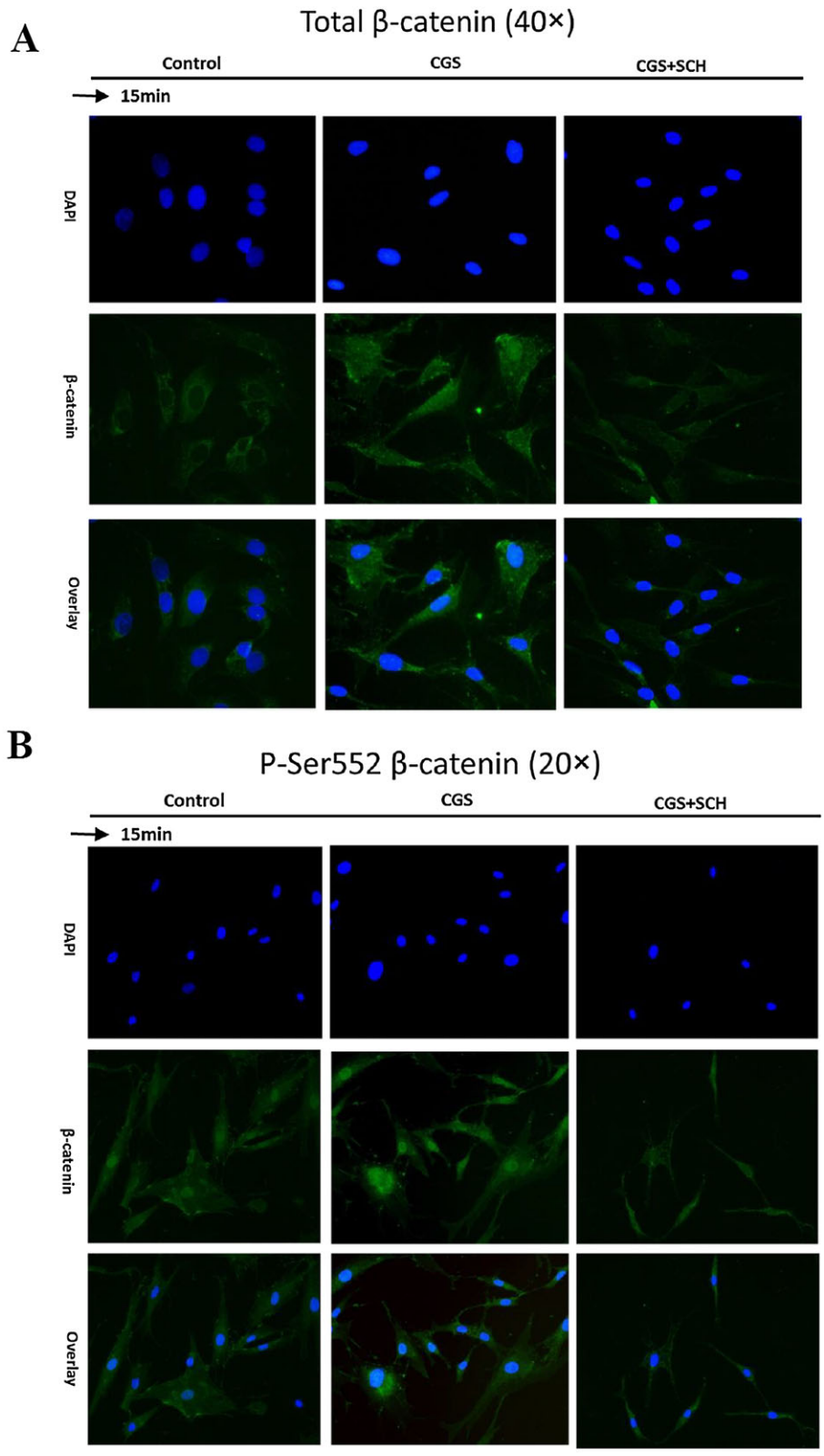

Figure 3

Nuclear translocation of β-catenin in NHDFs after CGS21680 (1 μM) stimulation for 15 min. NHDFs were processed and immunohistological staining was carried out. Nuclei are shown in blue (DAPI) and β-catenin in green. CGS21680 (1 μM) stimulation induced nuclear translocation of (A) total β-catenin, (B) p-Ser^{552} β-catenin and (C) de-phospho β-catenin. SCH58261 (1 μM) blocked the nuclear translocation of β-catenin induced by CGS21680 stimulation. CGS indicates CGS26180 and SCH indicates SCH58261.

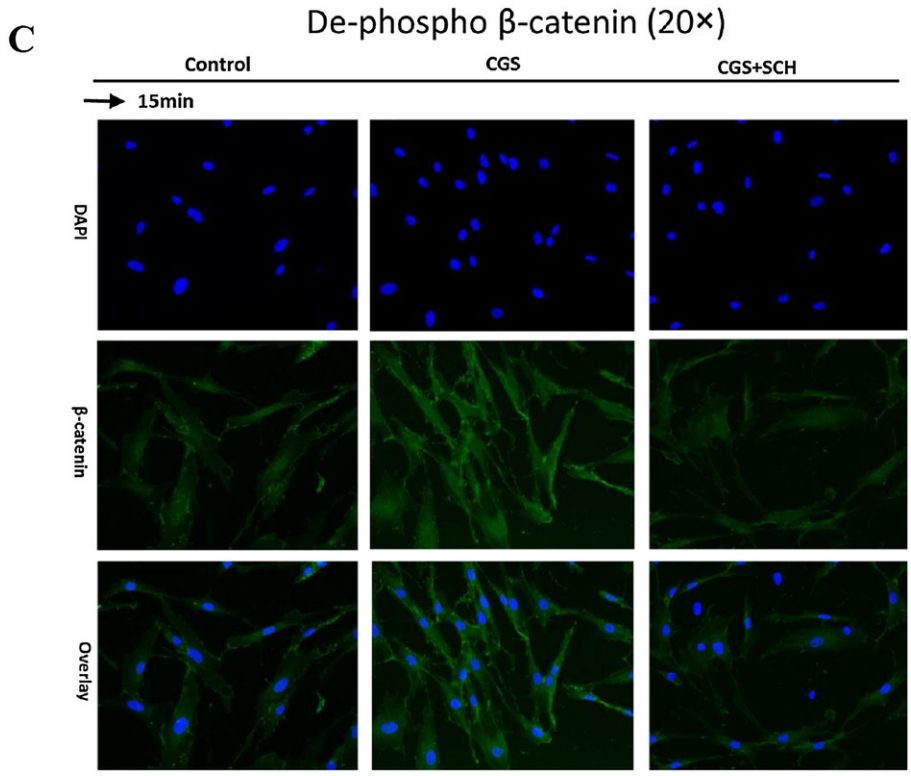

Figure 3
(Continued)

β-catenin activation by AKT, p-Ser^{552} β-catenin) after CGS21680 stimulation for time periods up to 4 h (Figure 2A).

After A_{2A} receptor stimulation by CGS21680 (1 μM), cellular p-Ser^{552} β-catenin levels rapidly increased more than two-fold at 15 min (220 ± 22% of non-stimulated control, $P < 0.05$, $n = 6$) and at 30 min (222 ± 36% of non-stimulated control, $P < 0.05$, $n = 6$) before gradually decreasing (Figure 2B). The point of maximum increase was consistent with that of total cellular β-catenin levels after CGS21680 stimulation (Figure 1A). A_{2A} receptor stimulation by CGS21680 (1 μM) also rapidly enhanced cellular de-phospho β-catenin levels, although this increase did not achieve statistical significance (Figure 2C). Our data indicate that activation of A_{2A} receptors increases cellular β-catenin levels via both canonical activation and non-canonical activation.

Activation of A_{2A} receptors induces nuclear translocation of β-catenin

Because activation of the Wnt/β-catenin signalling pathway ultimately results in the nuclear translocation of stabilized β-catenin, we next examined the β-catenin levels in the nuclear and cytoplasmic fraction of NHDFs after CGS21680 stimulation. Nuclear translocation of β-catenin was observed by fluorescence microscopy after CGS21680 (1 μM) (Figure 3A) stimulation and the nuclear translocation of both p-Ser^{552} β-catenin and de-phospho β-catenin was blocked by pretreatment with the A_{2A} receptor-selective antagonist SCH58261 (1 μM) (Figure 3B and C). We observed that, after CGS21680 (1 μM) stimulation, β-catenin levels in the cytosol fraction increased and peaked after 1 h (158 ± 36% of non-stimulated control, $P < 0.05$, $n = 5$). Thereafter, β-catenin levels in the cytosolic fraction dramatically decreased and this was accompanied by a concomitant increase in β-catenin levels in the nuclear fraction with a maximum at 2 h (151 ± 13% of non-stimulated control, $P < 0.05$, $n = 5$) (Figure 4A). Next, we examined the levels of active β-catenin in the nuclear fraction after 15 min of CGS21680 (1 μM) stimulation. There was a statistically significant increase in the levels of both de-phospho β-catenin (132 ± 10% of non-stimulated control, $P < 0.05$, $n = 5$) and p-Ser^{552} β-catenin (165 ± 8% of non-stimulated control, $P < 0.05$, $n = 5$) (Figure 4B). The increase in β-catenin levels in the nuclear fraction is consistent with the translocation of β-catenin from the cytosol to the nucleus after A_{2A} receptor activation. Taken together, these results demonstrate that activation of the A_{2A} receptor induces the nuclear translocation of β-catenin.

β-catenin increases Col3, but not Col1 synthesis after A_{2A} receptor activation

The results of previous studies have indicated that A_{2A} receptor activation increases the production of collagen in dermal fibroblasts (Chan *et al.*, 2013; Perez-Aso *et al.*, 2013). Because A_{2A} receptor activation increases cellular β-catenin levels and promotes its nuclear translocation for transcriptional activation, we further determined whether β-catenin signalling is involved in collagen production after A_{2A} receptor activation. After β-catenin had been reduced by use of siRNA (46 ± 5% decrease in β-catenin vs. scrambled siRNA transfection,

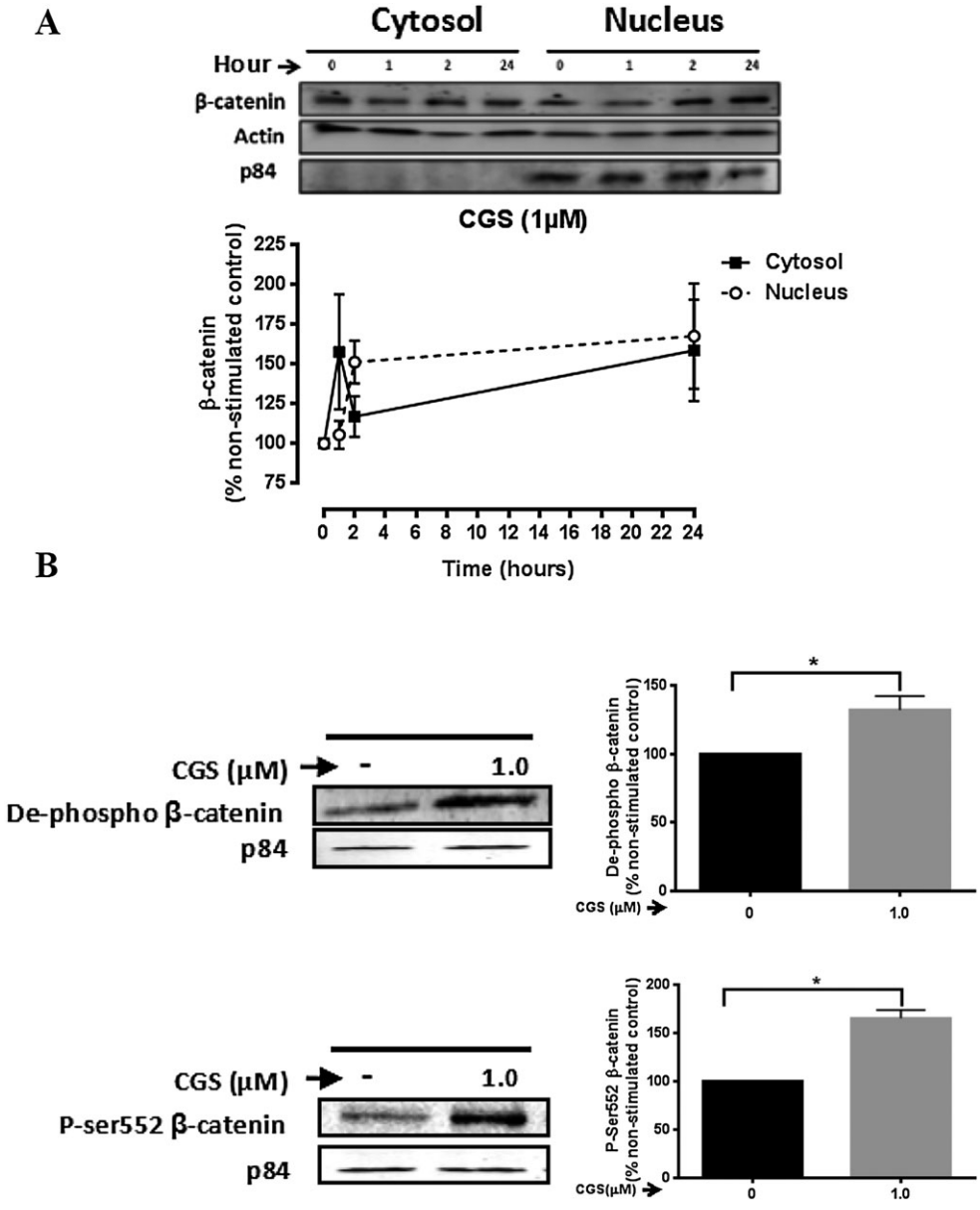

Figure 4

Stimulation of A_{2A} receptors leads to nuclear translocation of β-catenin. (A) NHDF cells were incubated with CGS21680 (1 μM) for periods up to 24 h and collected at the indicated time-points. Cytosolic and nuclear fractionation was performed as described in the Methods section. Representative images of western blots for β-catenin, actin and the nuclear marker p84 are shown. Effects of CGS21680 stimulation on β-catenin levels in the cytosol and nucleus were analysed. Data represent mean ± SEM of five independent experiments as determined by densitometry relative to actin or the nuclear marker p84. (B) NHDF cells were incubated with CGS21680 (1 μM) for 15 min. Representative images of western blots for de-phospho and p-Ser^{552} β-catenin in the nuclear fraction are shown. Effects of CGS21680 stimulation on de-phospho and p-Ser^{552} β-catenin levels in the nucleus were analysed. Data represent mean ± SEM of five independent experiments as determined by densitometry relative to the nuclear marker p84. *$P < 0.05$ versus non-stimulated control. CGS indicates CGS26180.

$P < 0.05$, $n = 10$) (Figure 5A), we observed that the increase in Col1 synthesis induced by A_{2A} receptor stimulation by CGS21680 (1 μM) was unaffected by β-catenin knockdown (scrambled siRNA 63 ± 22% increase of Col1 vs. β-catenin-siRNA 53 ± 17% increase of Col1, $P > 0.05$, $n = 7$). In contrast, β-catenin knockdown inhibited A_{2A} receptor-mediated Col3 synthesis by 73% (scrambled siRNA 66 ± 14% increase of Col3 vs. β-catenin siRNA 18 ± 16% increase of Col3, $P < 0.05$, $n = 8$) (Figure 5B). These results indicate that β-catenin signalling is involved in the synthesis of Col3 but not Col1 evoked by activation of A_{2A} receptors.

Discussion and conclusions

In the present study we showed that the stimulation of A_{2A} receptors rapidly increases total cellular β-catenin levels via both canonical and Akt-mediated activation. Moreover, consistent

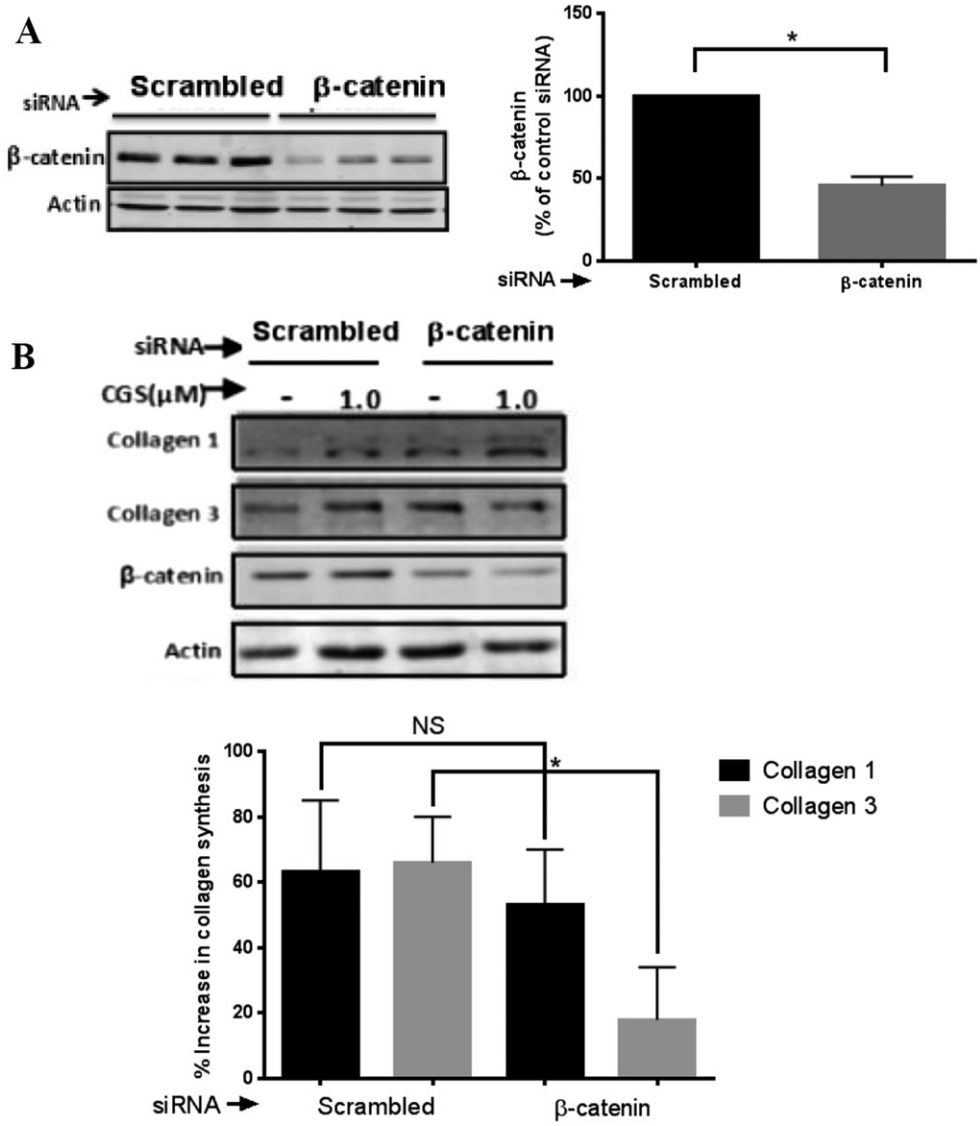

Figure 5

β-Catenin knockdown prevents A_{2A} receptor-mediated increase in Col3 but not Col1. NHDF cells were stably transfected with scrambled siRNA or β-catenin siRNA. (A) Cellular β-catenin levels were measured. Representative western blots of β-catenin and actin are shown. Data represent mean ± SEM of 10 independent experiments as determined by densitometry relative to actin. (B) CGS21680 (1 μM) was added for 24 h, after β-catenin knockdown. A_{2A} receptor stimulation increased Col1 synthesis and was unaffected by β-catenin knockdown. In contrast, A_{2A} receptor-mediated increase in Col3 synthesis was reduced with β-catenin knockdown. Representative western blots of Col1, Col3, β-catenin and actin are shown. Data represent mean ± SEM of seven (Col1) and eight (Col3) independent experiments as determined by densitometry relative to actin. NS, $P > 0.05$; *$P < 0.05$, β-catenin siRNA versus scrambled siRNA. CGS indicates CGS26180.

with its effects on cellular β-catenin levels, activation of the A_{2A} receptor leads to the nuclear translocation of both phosphorylated serine-552 and de-phosphorylated β-catenin at 15 min. Subsequently, there is a further increase in this nuclear translocation up to a maximum of 24 h consistent with the previously reported biphasic nuclear shuttling of β-catenin following Wnt signalling (Xie *et al.,* 2008; Zhang *et al.,* 2011; Jang *et al.,* 2014; Tapia-Rojas *et al.,* 2015). The finding that silencing the expression of β-catenin prevents the synthesis of Col3 mediated by A_{2A} receptors, but not that of Col1, sheds light on the signalling downstream from the A_{2A} receptor involved in stimulating fibrosis and scarring.

Adenosine, by acting on A_{2A} and A_{2B} receptors, increases both Col1 and Col3 synthesis *in vitro* and in animal models (Perez-Aso *et al.,* 2013, 2014). Adenosine receptors are differentially expressed in different tissues and signal via distinct pathways downstream from the secondary messenger cAMP (Shaikh and Cronstein, 2016). For example, the A_{2B} receptor mediates renal fibrosis in the setting of hypoxia by inducing the expression of endothelin-1 (Sorokin and Kohan, 2003; Kong *et al.,* 2006). This pathway is also important in promoting pulmonary interstitial disease (Karmouty-Quintana *et al.,* 2012). The A_{2A} receptor is the principal adenosine receptor involved in dermal fibrosis (Chan *et al.,* 2006; Perez-Aso

et al., 2013). In normal skin, the ratio of Col1 to Col3 is approximately 4:1; however, in granulation tissue and immature scars, where local adenosine concentrations are elevated, the increased synthesis of Col3 leads to a reduction in the Col1 to Col3 ratio to 2:1 (Perez-Aso *et al.,* 2013). High levels of A_{2A} receptor stimulation, with correspondingly higher cAMP concentrations, lead to the induction of Epac2 and promote Col3 synthesis (Perez-Aso *et al.,* 2013). In addition, A_{2A} receptor ligation stimulates an increase in the secretion of connective tissue growth factor (CTGF), which promotes collagen secretion by diminishing the expression of the transcriptional regulator Fli1, a constitutive repressor of CTGF expression (Chan *et al.,* 2013).

The results presented here demonstrate crosstalk between A_{2A} receptors and Wnt/β-catenin signalling, which probably occurs at several levels (Figure 6). For example, Fli1 inhibits β-catenin/TCF-mediated transcription, and CTGF leads to the accumulation and nuclear translocation of β-catenin as well as increased TCF/LEF transcriptional activity (Navarro *et al.,* 2010; Rooney *et al.,* 2011). PKA can also directly stabilize β-catenin by inhibiting its ubiquitination (Hino *et al.,* 2005). Downstream, PKA activates cAMP response element-binding protein (CREB) by phosphorylation, which can modulate gene expression directly through interaction with gene promoters or indirectly by competing with NFκB and other transcription factors for the cofactor CREB-binding protein (CBP) (Hasko *et al.,* 2008). CBP is a transcriptional co-activator of β-catenin, and CBP/β-catenin-mediated transcription is critical for cellular proliferation (Hasko *et al.,* 2008; Henderson *et al.,* 2010). Furthermore, selective inhibition of the CBP/β-catenin interaction attenuates bleomycin-induced lung fibrosis and reverses established fibrosis (Takemaru and Moon, 2000; Henderson *et al.,* 2010).

At low levels of A_{2A} receptor activation, PKA represses Col3 synthesis. However, interestingly, at higher levels of receptor engagement, with a correspondingly higher level of cAMP, Col3 levels are increased. This is probably due to the induction of Epac2 and the positive cooperativity of cAMP binding to PKA (Perez-Aso *et al.,* 2013). We hypothesize that if the A_{2A} receptor/PKA/β-catenin pathway is important for Col3 synthesis this reflects PKA's unique role as a molecular switch that can integrate multiple upstream signals and can activate different downstream pathways depending on cell-specific conditions.

It has also been shown that the effects of A_{2A} receptor stimulation on Col3 synthesis are, in part, mediated by p38-MAPK (Perez-Aso *et al.,* 2013). Activation of p38-MAPK also interacts with canonical Wnt/β-catenin signalling (Bikkavilli *et al.,* 2008). However, although inhibition of p38-MAPK interrupts Wnt/β-catenin signalling, its knockdown does not completely abolish the signalling. Rather, p38-MAPK activation operates as a parallel pathway feeding into the Wnt/β-catenin pathway by inhibiting GSK3β, a key regulatory enzyme in canonical signalling (Bikkavilli *et al.,* 2008). Finally, direct phosphorylation of β-catenin at the serine-552 residue by Akt promotes its transcriptional activity without altering its stability and phosphorylation level by GSK3β. Thus, A_{2A} receptor ligation

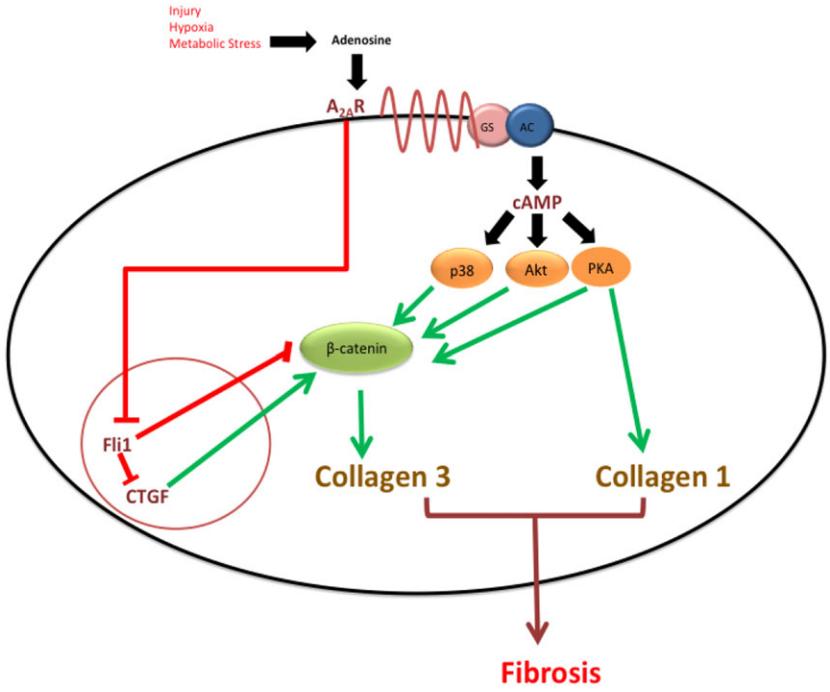

Figure 6

Crosstalk between A_{2A} receptors and Wnt/β-catenin signalling pathways in human dermal fibroblasts. Adenosine levels increase by several orders of magnitude during cellular injury, hypoxia or metabolic stress. Stimulation of A_{2A} receptors activates a Gs protein leading to a rise in cAMP via adenylyl cyclase and activation of downstream targets. MAPK-p38, PKA and CTGF have all been shown to directly promote canonical β-catenin signalling, and Fli1 represses canonical β-catenin signalling. Akt, which activates β-catenin non-canonically at Ser^{552}, also promotes its transcriptional activity (see text). Together, these pathways promote normal wound healing but also pathological fibrosis via Col3 synthesis.

represents another parallel, non-canonical pathway for activation of β-catenin (Fang *et al.,* 2007).

Our results show that β-catenin activation and translocation are necessary for A_{2A} receptor-mediated Col3 synthesis but not Col1 synthesis. Interestingly, β-catenin signalling is required for Smad-dependent TGF-β1-induced Col1 but not Col3 synthesis (Baarsma *et al.,* 2011). Although both TGF-β1 and adenosine receptors can induce Col1 and Col3 synthesis, we hypothesize that A_{2A} receptor-dependent β-catenin signalling is preferentially activated in early wound healing and granulation tissue formation, whereas TGF-β1-mediated β-catenin signalling may be involved in scar maturation.

The A_{2A} receptor is also likely to interact with Wnt/β-catenin signalling in other tissues. For example, in bone, A_{2A} receptor activation inhibits the differentiation and function of osteoclasts (Mediero *et al.,* 2013). As a consequence, A_{2A} receptor-deficient mice have markedly increased numbers of osteoclasts and increased bone resorption with diminished bone density (Mediero *et al.,* 2013). Similarly, in mature osteoblasts and osteocytes, β-catenin plays a role in suppressing osteoclast differentiation (Chen and Long, 2013). In humans, loss-of-function mutations of β-catenin lead to early onset osteoporosis (Chen and Long, 2013). One pathway by which A_{2A} receptor stimulation regulates bone turnover is via PKA-dependent inhibition of the nuclear translocation of NFκB (Mediero *et al.,* 2013). Activation of PKA directly stabilizes β-catenin and has been shown to inhibit NFκB activation and translocation to the nucleus through a physical interaction (Takahashi *et al.,* 2002). Similarly, the PI3-kinase-Akt signalling pathway, previously shown to activate β-catenin in a non-canonical fashion, facilitates osteoblast differentiation, bone growth and mineralization (Saidak *et al.,* 2015).

In conclusion, we have shown that A_{2A} receptor activation leads to activation of both canonical and non-canonical Wnt/β-catenin signalling, which is required for Col3 but not Col1 synthesis in primary human dermal fibroblasts. The molecular crosstalk between these two signalling pathways probably occurs at multiple levels in different tissues. Wnt/β-catenin signalling also represents a final common pathway for both TGF-β1 and A_{2A} receptor signalling pathways, both of which are essential in wound healing and fibrosis. Thus, selectively modifying this pathway represents an attractive therapeutic target in fibrotic diseases.

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Author contributions

G.S., J.Z., M.P-A., A.M. and B.C. contributed to the conception and design of the experiments. G.S., J.Z., A.M. and M.P-A. conducted the experiments, collected and assembled the data, and performed data analysis and interpretation. G.S., J.Z. and B.C. contributed to manuscript preparation.

Conflict of interest

A.M. and B.C. have filed a patent on use of adenosine A2AR agonists to prevent prosthesis loosening (pending). A.M. and B.C. have filled a patent on the use of Antibodies against Netrin-1 for the treatment of bone diseases. G.S., J.Z. M.P-A. do not have any disclosures. B.C. holds patents numbers 5,932,558; 6,020,321; 6,555,545; 7,795,427; adenosine A1R and A2BR antagonists to treat fatty liver (pending); adenosine A2AR agonists to prevent prosthesis loosening (pending). B.C. is a consultant for Bristol-Myers Squibb, AstraZeneca, Novartis, CanFite Biopharmaceuticals, Cypress Laboratories, Regeneron (Westat, DSMB), Endocyte, Protalex, Allos, Inc., Savient, Gismo Therapeutics, Antares Pharmaceutical, Medivector, King Pharmaceutical, Celizome, Tap Pharmaceuticals, Prometheus Laboratories, Sepracor, Amgen, Combinatorx, Kyowa Hakka, Hoffman-LaRoche and Avidimer Therapeutics. BNC has stock in CanFite Biopharmaceuticals.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organisations engaged with supporting research.

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