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Themed Section: Novel cAMP Signalling Paradigms

REVIEW

cAMP and Epac in the regulation of tissue fibrosis

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Fibrosis, the result of excess deposition of extracellular matrix (ECM), in particular collagen, leads to scarring and loss of function in tissues that include the heart, lung, kidney and liver. The second messenger cAMP can inhibit the formation and extent of ECM during this late phase of inflammation, but the mechanisms for these actions of cAMP and of agents that elevate tissue cAMP levels are not well understood. In this article, we review the fibrotic process and focus on two recently recognized aspects of actions of cAMP and its effector Epac (Exchange protein activated by cAMP): (a) blunting of epithelial–mesenchymal transformation (EMT) and (b) down-regulation of Epac expression by profibrotic agents (e.g. TGF-β, angiotensin II), which may promote tissue fibrosis by decreasing Epac-mediated antifibrotic actions. Pharmacological approaches that raise cAMP or blunt the decrease in Epac expression by profibrotic agents may thus be strategies to block or perhaps reverse tissue fibrosis.

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Abbreviations
CF, cardiac fibroblast; ECM, extracellular matrix; Epac, Exchange protein activated by cyclic AMP; 8-Me-cAMP, 8-CPT-2′-O-Me-cAMP; N6-cAMP, N6-Benzoyl-cAMP; NSAIDs, non-steroidal anti-inflammatory drugs; α-SMA, α-smooth muscle actin

Introduction to tissue fibrosis and cAMP signalling

Tissue fibrosis

Tissue fibrosis (scarring) results from the excess deposition of extracellular matrix (ECM) and occurs as part of normal physiology (e.g. aging) and following injury, in particular, with recurrent or persistent stimulation of the inflammatory process. Tissue fibrosis often alters tissue function. For example, cardiac fibrosis contributes to diastolic dysfunction and a decrease in cardiac output that accompany advanced age (Lakatta, 2003; Chen and Frangogiannis, 2010). Inflammation that follows tissue injury produces a series of acute and late-phase responses. In its acute phase, inflammation is characterized by several cardinal features [calor, rubor, dolor and tumour (heat, redness, pain and swelling)], which can be treated by pharmacological agents, in particular non-steroidal anti-inflammatory drugs (NSAIDs), whose major action is inhibition of COXs and thus the synthesis of prostaglandins.

Much less is known regarding the mechanisms that mediate the late phase of inflammation during which resolution of the tissue infiltration of acute inflammatory cells occurs, but tissue fibrosis can be initiated. Recent efforts have helped identify mechanisms of the resolution of inflammation (Serhan, 2010; Maskrey *et al.*, 2011; Wynn, 2011; Yates *et al.*, 2011). Glucocorticoids, the principal class of drugs used to treat this phase of the inflammatory process, block protein synthesis, thereby decreasing the accumulation of collagens and other ECM proteins. However, the use of glucocorticoids is associated with numerous side effects. Pirfenidone is a

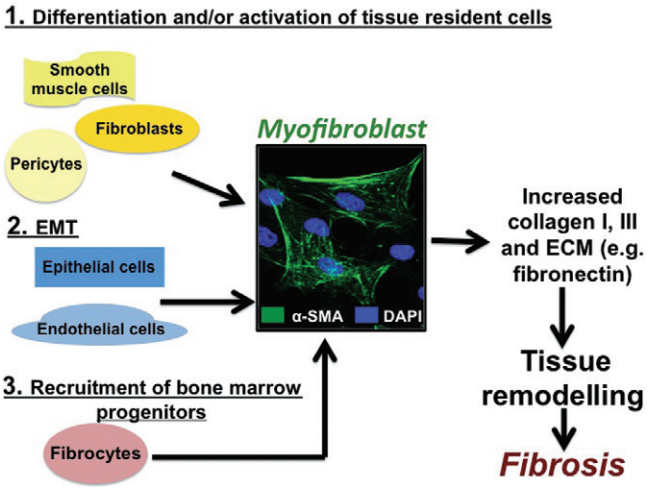

Figure 1

Heterogeneous origin of myofibroblasts. Myofibroblasts, which synthesize and secrete ECM, including collagens I and III, and contribute to fibrosis, originate from various sources, which include the following:
(1) Differentiation of fibroblasts, activation of pericytes (e.g. hepatic stellate cells) and de-differentiation of muscle cells;
(2) EMT or endothelial–mesenchymal transformation (transition);
(3) Recruitment of circulating fibrocytes derived from the bone marrow.

recently available antifibrotic agent whose mechanism of action is, at least in part, inhibition of the production and activity of TGF-β, a cytokine with strong profibrotic actions (Cho and Kopp, 2010). The wide prevalence of diseases associated with tissue fibrosis and the increasing age of the population (which is accompanied by such disorders) provide a strong impetus for the discovery of new antifibrotic agents.

A key goal in developing treatments for fibrosis is the avoidance of tissue damage and resolution of the remodelling that occur as a consequence of deposition of ECM with a resultant decrease in tissue parenchyma and function. Although certain types of tissue fibrosis can be reversed, it is generally irreversible, especially if its extent produces substantial loss of tissue parenchyma (Wallace *et al.*, 2007; Marian, 2009; Ramachandran and Iredale, 2009; Chen and Frangogiannis, 2010; Dussaule *et al.*, 2011). The main cell responsible for tissue remodelling during fibrosis is the myofibroblast. Myofibroblasts, α-smooth muscle actin–expressing cells that secrete ECM components and collagen type I and III, originate from a number of sources that depend on the physiological stimulus and the tissue in which the insult occurs. The mechanisms that contribute to the increase in the number of myofibroblasts in fibrosis are outlined in Figure 1 and include (Krenning *et al.*, 2010; Pinzani and Macias-Barragan, 2010; Meran and Steadman, 2011) (1) mitosis of tissue-resident fibroblasts and their phenotypic 'switch' to profibrotic myofibroblasts (Hinz, 2010); (2) altered stem cell differentiation or de-differentiation of other cell types into fibroblasts (Brack *et al.*, 2007); (3) activation and migration of stromal cells, pericytes, particularly in kidney fibrosis (Armulik *et al.*, 2011; Schrimpf and Duffield, 2011); (4) conversion of epithelial or endothelial cells to fibroblasts, via epithelial–mesenchymal transformation (termed EMT) or endothelial–mesenchymal transformation, respectively; and (5) entry of bone marrow-derived, circulating fibrocytes into inflammatory sites (Grieb *et al.*, 2011). The relative contribution of these various mechanisms to fibrosis is a topic of considerable current debate along with the question as to whether myofibroblasts derived in different ways and from different tissues exhibit different characteristics and functions; since myofibroblasts produce mediators that contribute to the fibrotic process, attenuation of their recruitment and activation are approaches to treat tissue fibrosis (Hinz, 2010; Humphreys *et al.*, 2010; Krenning *et al.*, 2010; Pinzani and Macias-Barragan, 2010; Kriz *et al.*, 2011; Meran and Steadman, 2011).

cAMP signalling

The second messenger cAMP is as a regulator of fibroblast function. cAMP is generated by ACs in response to activation of GPCRs and degraded by cyclic nucleotide PDEs. cAMP acts via three mechanisms to modulate tissue responses: PKA, Epac and cyclic nucleotide-gated (CNG) ion channels. The cAMP effector, Epac (Exchange protein activated by cAMP) (Gloerich and Bos, 2010; Grandoch *et al.*, 2010; Breckler *et al.*, 2011), has recently been identified as an important mediator of the antifibrotic effect of cAMP and will be discussed in more detail below. As reviewed in the references cited above, the two forms of Epac, Epac1 and Epac2, respectively possess one and two cAMP binding sites and function as guanine nucleotide exchange factors for the low molecular weight G-protein Rap (e.g. Rap1 and Rap2). Epac proteins regulate numerous cellular responses through their ability to promote the exchange of GTP for GDP on Raps and perhaps other G-proteins (Gloerich and Bos, 2010; Grandoch *et al.*, 2010; Breckler *et al.*, 2011). cAMP analogues that selectively activate PKA or Epac (Bos, 2006; Holz *et al.*, 2008) can aid in defining their role as effectors of cellular responses.

Increases in cAMP levels inhibit fibroblast function and produce antifibrotic effects that include (Table 1) decrease in fibroblast proliferation, stimulation of the death of fibroblasts and inhibition of ECM protein synthesis. Such effects can occur in response to increases in cAMP by agonists/antagonists of GPCRs, activation of ACs, inhibition of PDEs or use of cAMP analogues. The precise mechanisms for the antifibrotic effects of cAMP that are summarized in Table 1 are not clearly defined. We will discuss two aspects of cAMP/Epac-regulated actions that influence tissue fibrosis: (1) inhibition by cAMP of EMT and an associated decrease in synthesis of ECM components by fibroblasts and (2) inhibition by profibrotic agents of Epac expression, thereby blunting its antifibrotic actions. In addition, we discuss the roles of cAMP effectors in fibrosis and provide some therapeutic implications of these actions.

Myofibroblast transformation is inhibited by cAMP via Epac and PKA

EMT

EMT has been proposed as a mechanism for tissue fibrosis in numerous settings, including in development, cancer and

Table 1

Antifibrotic actions of cAMP

Action	References	Cell type
Inhibition of formation and action of profibrotic growth factors/hormones	Windmeier and Gressner, 1997	Rat hepatic fibroblasts
	Kothapalli *et al.*, 1998	Rat kidney interstitial fibroblasts (NRK-49F)
	Heusinger-Ribeiro *et al.*, 2001	Immortalized human renal fibroblasts
	Yu *et al.*, 2002	Rat-1 Fibroblasts
	Swaney *et al.*, 2005	Rat cardiac fibroblasts
	Liu *et al.*, 2006	Rat cardiac fibroblasts
	Black *et al.*, 2007	Human gingival fibroblasts
	Clancy *et al.*, 2007	Human Fetal Cardiac Fibroblasts
Decrease in fibroblast proliferation and fibroblast –> myofibroblast conversion	Dunkern *et al.*, 2007	Human lung fibroblasts, fibroblast cell line IMR-90, fetal human fibroblast cell line GM06114
	Huang *et al.*, 2008	Human fetal lung fibroblast cell line IMR-90, primary human lung fibroblasts
	Xing and Bonanno, 2009	Primary rabbit corneal keratocytes
	Sandbo *et al.*, 2009	Human pulmonary fibroblasts
	Selige *et al.*, 2010	Human lung fibroblasts
	Liu *et al.*, 2004; 2005; 2010	Rat pulmonary fibroblasts, murine pulmonary fibroblasts
	Stumm *et al.*, 2011	Murine lung fibroblasts
Promotion of cell death of fibroblasts	Huang *et al.*, 2009	Human fetal lung fibroblast cell line IMR-90, primary human lung fibroblasts
	Insel *et al.*, 2012b	Various
	Zhang *et al.*, 2011	Rat cardiac fibroblasts
Decrease in fibroblast motility	Sandulache *et al.*, 2006; 2007	Human fetal dermal fibroblast, human adult dermal fibroblast
	Yokoyama *et al.*, 2008	Rat cardiac fibroblasts
	Togo *et al.*, 2009	Human fetal lung fibroblasts (HFL-1)
	Kohyama *et al.*, 2009	Human fetal lung fibroblasts (HFL-1)
Decrease in synthesis/ release/function of ECM components	Parekh *et al.*, 2007	Human fetal dermal fibroblast, human adult dermal fibroblast
	Sachs *et al.*, 2007	Murine lung fibroblasts
	Liu *et al.*, 2008	Rat cardiac fibroblasts
	Parekh *et al.*, 2009	Human fetal dermal fibroblast, human adult dermal fibroblast
	Huang *et al.*, 2008	Human fetal lung fibroblast cell line IMR-90, primary human lung fibroblasts
	Liu *et al.*, 2004; 2005; 2010	Rat pulmonary fibroblasts, murine pulmonary fibroblasts
	Bauman *et al.*, 2010	Murine lung fibroblasts, human fetal lung fibroblast cell line IMR-90
	Chan *et al.*, 2010	Murine cardiac fibroblasts
	D'Souza *et al.*, 2011	Human cardiac fibroblasts
	Okunishi *et al.*, 2011	Murine lung fibroblasts
	Miller *et al.*, 2011	Murine and rat fibroblasts

diseases of many organs (Hinz, 2010; Humphreys *et al.*, 2010; Krenning *et al.*, 2010; Chaffer and Weinberg, 2011; Kriz *et al.*, 2011; Meran and Steadman, 2011). A commonly used model to study EMT is to ligate one of the ureters and observe the resultant changes in the kidney. Other studies of EMT have used renal epithelial cells, such as the distal tubule/collecting duct-derived MDCK cells. MDCK cells treated with TGF-β show changes that occur with EMT (Zhang *et al.*, 2006a,b; Park *et al.*, 2007; Cufí *et al.*, 2010; Li *et al.*, 2010; Figure 2): loss of expression of E-cadherin (and other proteins characteristic of epithelial cells) and increased expression of α-smooth muscle actin (α-SMA) [and also vimentin, fibronectin and collagens (proteins characteristic of fibroblasts; data not shown)]. We observe such changes in parental MDCK cells treated with TGF-β but not in MDCK-D1 cells, a clonal isolate that has enriched expression of α_{1b}- and β_2-adrenoceptors (Klijn *et al.*, 1991; data not shown). Parental MDCK cells represent a mixture of renal cell types; an extensive literature

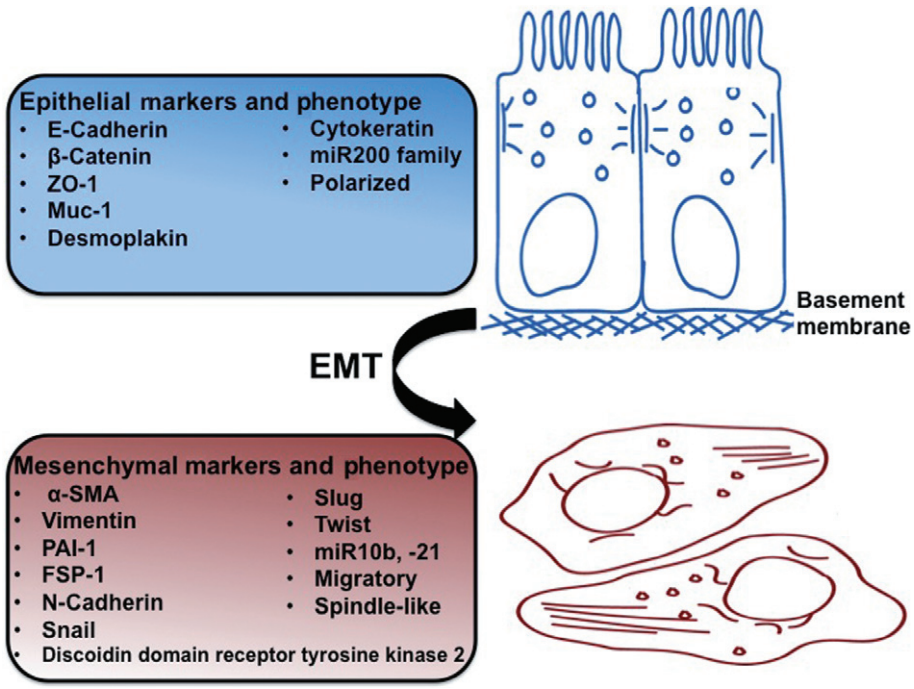

Figure 2

Markers of EMT. EMT involves a transition of polarized epithelial cells, which normally interact with the basement membrane, into migratory spindle-like, ECM-producing mesenchymal cells. The figure lists some commonly used markers that distinguish the two phenotypes.

documents that clonal isolates can have properties that differ from those present in the parental cells (e.g. Meier *et al.*, 1983).

Increases in cAMP by agents that activate AC, PDE inhibitors or cAMP analogues inhibits EMT in MDCK cells (Zhang *et al.*, 2006a,b; Figures 3 and 4). Based on their epithelial phenotype, parental MDCK cells have high expression of E-cadherin but express little or no α-SMA: TGF-β induces transition to a mesenchymal phenotype by increasing α-SMA and decreasing E-cadherin expression (Figures 3 and 4). Treatment of these MDCK cells with a cAMP derivative that selectively activates Epac (8-Me-cAMP, 50 μM) but not with a PKA-selective cAMP agonist (N6-cAMP, 50 μM) blunts the increase in α-SMA (Figures 3 and 4). By contrast, both 8-Me-cAMP and N6-cAMP attenuate the TGF-β-promoted inhibition of E-cadherin expression. Thus, inhibition of aspects of EMT by cAMP in MDCK cells involves both Epac and PKA.

Fibroblast-myofibroblast transformation

Studies with adult rat cardiac fibroblasts (CF) reveal that increasing cAMP formation by the overexpression of AC6 or by activating AC with forskolin blunts collagen synthesis, TGF-β- or angiotensin II-stimulated increase in α-SMA expression, actin/focal adhesion assembly and, in parallel, the transformation of fibroblasts to myofibroblasts (Swaney *et al.*, 2005). Experiments conducted with N6-cAMP and 8-Me-cAMP indicate that activation of either PKA or Epac inhibit expression of collagens I and III; by contrast, Epac and PKA have opposing effects on CF migration (Yokoyama *et al.*, 2008). Such data are consistent with results observed with other fibroblasts, such as those from the lung (Huang *et al.*, 2008).

Regulation of Epac expression by profibrotic agents

Our studies with MDCK cells (Figure 5) and primary cultures of rat cardiac fibroblasts (Yokoyama *et al.*, 2008) reveal that treatment with profibrotic agents decreases the expression of Epac, results confirmed by others (Basoni *et al.*, 2005). Prevention of this decrease in Epac expression inhibits profibrotic response (Yokoyama *et al.*, 2008), thus implying the importance of the decrease in Epac for this response.

Treatment of MDCK cells with profibrotic agents decreases expression of Epac2, which is expressed at much higher levels than Epac1 in these cells (Figure 5), but in fibroblasts from multiple tissues, profibrotic agents only decrease expression of Epac1 (Yokoyama *et al.*, 2008). Thus, profibrotic agents can down-regulate expression of both isoforms of Epac and Epac-mediated signalling, such as activation of Rap-1, but alter expression of different isoforms in different tissues.

The roles of cAMP and Epac in tissue fibrosis and therapeutic implications

The information provided above implies that therapeutic approaches that increase cAMP or that activate Epac or increase its expression have the potential to decrease tissue fibrosis (Figure 6). Although PKA was initially thought to be the exclusive mediator of cAMP action (Insel *et al.*, 1975), Epac mediates numerous effects of cAMP (Gloerich and Bos,

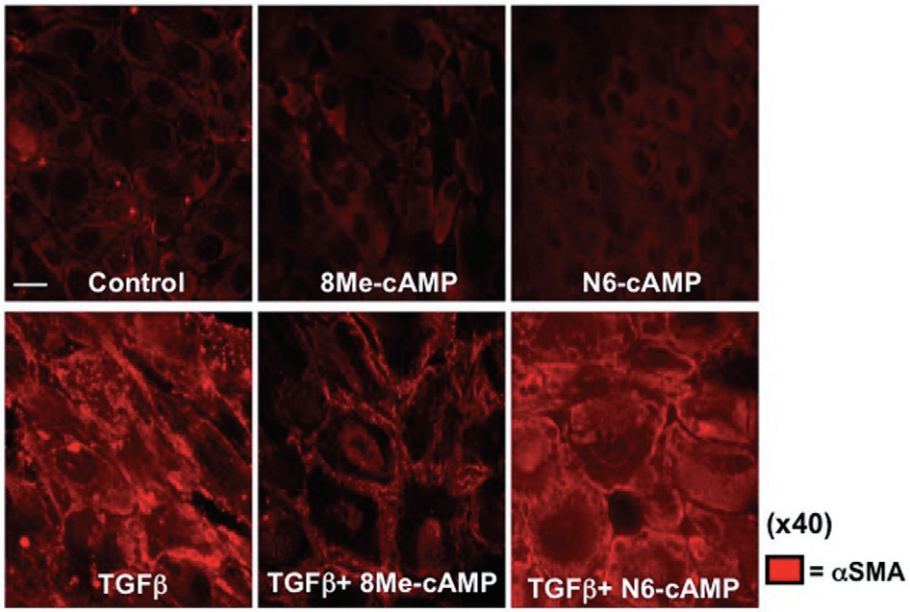

Figure 3

Activation of Epac, but not PKA, attenuates the TGF-β-induced increase in α-SMA in MDCK cells. Immunocytochemistry demonstrates that Epac activation (8Me-cAMP, 50 μM, 72 h), but not PKA activation (N6-cAMP, 50 μM, 72 h) inhibits the increase in α-SMA (stained red) induced by TGF-β1 (5ng·mL^{-1}, 72 h), 40× objective. Data are representative of three separate experiments. Bar equals 10 μm.

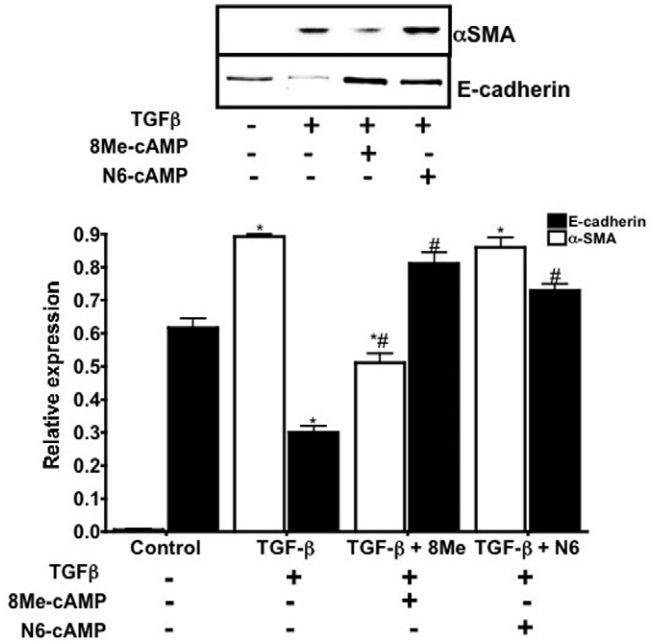

Figure 4

Activation of either Epac or PKA reverses characteristics of EMT in MDCK cells. Representative immunoblot reveals that MDCK cells treated with TGF-β1 (5ng·mL^{-1}, 72 h) show changes associated with EMT (increased α-SMA and loss of E-cadherin). Epac activation (8Me-cAMP, 50 μM, 72 h) inhibits the increase in α-SMA and the decrease in E-cadherin, whereas PKA activation (N6-cAMP, 50 μM, 72 h) only prevents the decrease in E-cadherin. *Lower panel*, immunoblots were quantified and normalized to GAPDH expression. $n = 3$; $^{*}P < 0.05$ versus control; $^{\#}P < 0.05$, versus TGF-β.

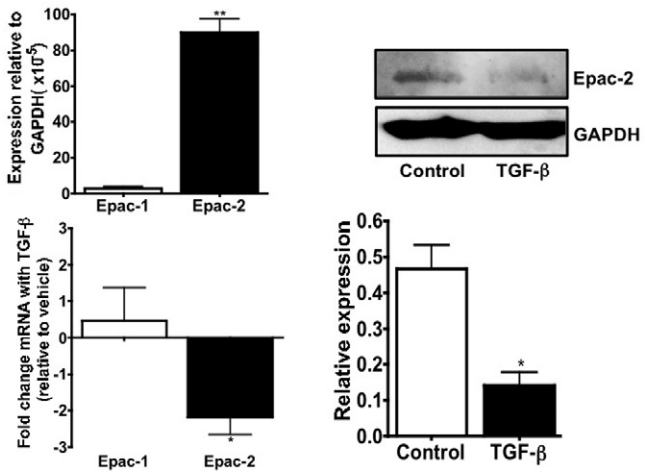

Figure 5

Epac2 expression is markedly decreased in MDCK cells by TGF-β. *Upper left panel*, Epac-1 and Epac2 mRNA expression in MDCK cells (by real-time PCR, results normalized to GAPDH) shows that Epac2 is expressed at higher levels than is Epac1 $n = 3$; $^{**}P < 0.001$ compared with Epac1. *Lower left panel*, treatment with 5 ng·mL^{-1} TGFβ1 for 72 h decreases Epac2 mRNA expression (by real-time PCR) $n = 3$; $^{*}P < 0.05$ versus vehicle. The data are expressed as fold change relative to vehicle-treated MDCKs. *Upper right panel*, a representative immunoblot shows that Epac2 protein expression is decreased in MDCK cells treated with 5 ng·mL^{-1} TGF-β1 for 72 h. *Lower right panel*, immunoblots were quantified and normalized to GAPDH expression. $n = 3$; $^{*}P < 0.05$, versus vehicle.

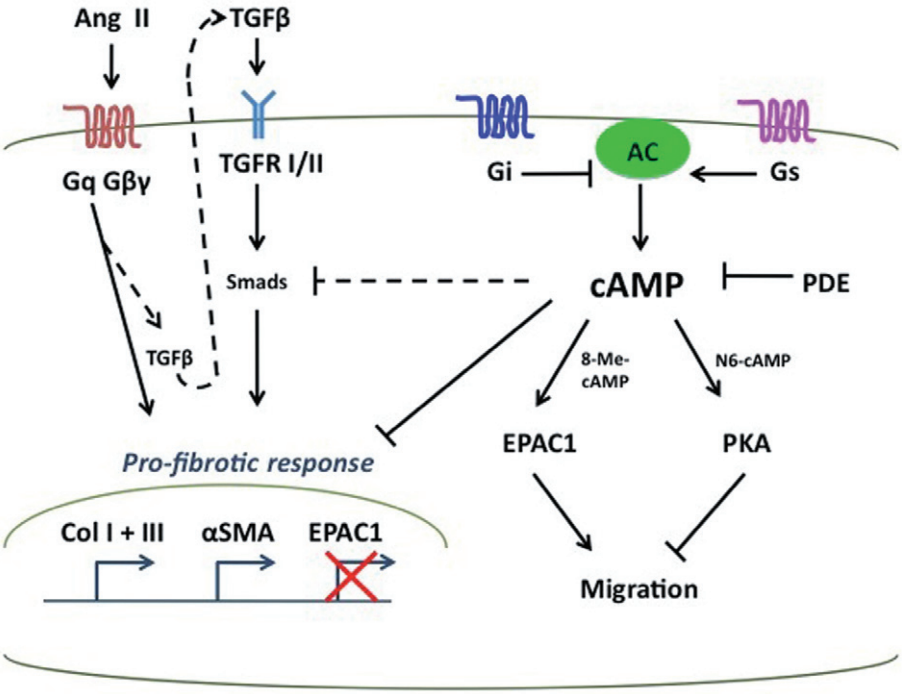

Figure 6

Model of the antifibrotic effects of cAMP. After injury, profibrotic stimuli (e.g. Ang II and TGF-β1) activate fibroblasts, increase ECM synthesis and deposition (e.g. collagens I and III), and regulate expression of fibrotic genes (e.g. α-SMA). Pharmacological agents that increase intracellular cAMP (Gs-linked GPCR agonists, Gi-linked GPCR antagonists, AC activators, or PDE inhibitors) can inhibit the profibrotic effects of Ang II and TGF-β1, potentially through SMAD inhibition. cAMP activation of Epac1 and PKA lowers expression of collagens I and III but has opposite effects on cell migration.

2010; Grandoch *et al.*, 2010; Breckler *et al.*, 2011). One such effect is the inhibition of EMT, although both PKA and Epac regulate features characteristic of EMT in MDCK cells (Figures 3 and 4). The contribution of EMT to tissue fibrosis *in vivo* is controversial (Hinz, 2010; Humphreys *et al.*, 2010; Kriz *et al.*, 2011; Quaggin and Kapus, 2011). Even so, the activation of Epac, potentially either Epac1 or Epac2 in different tissues, can regulate profibrotic responses, including collagen and DNA synthesis and other functional activities of fibroblasts. Data in rat cardiac, lung and skin fibroblasts show Epac1 is preferentially decreased in response to profibrogenic agonists; however, Epac2 is decreased by the same stimuli in MDCK cells (Figure 5; Yokoyama *et al.*, 2008). Other data implicate increases in cAMP as a means to inhibit EMT (Zhang *et al.*, 2006a,b; Kim *et al.*, 2009; Kolosionek *et al.*, 2009) and of PKA-mediated phosphorylation to influence proteins that regulate EMT (MacPherson *et al.*, 2010).

Recent information emphasizes the contribution of various transcription factors (e.g. Snail, Slug, Twist, etc.) to EMT and mechanisms that regulate their expression. EMT is thought to play an important role in pathophysiology in cancer and cancer cell metastases, with EMT-associated features representing signals expressed after the formation of primary tumours (Chaffer and Weinberg, 2011). The contribution of EMT to tissue fibrosis is controversial in a number of other settings, in part because not all features of the 'EMT phenotype' are consistently observed, especially between *in vitro* and *in vivo* models. A 'two-hit' model has been proposed that involves (1) disruption of intercellular contact between epithelial cells and (2) promotion of fibrotic features by TGF-β (Masszi *et al.*, 2010). A consequence of the disruption of intercellular contact is the nuclear translocation of myocardin-related transcription factor (MRTF), which binds to a region in the α-SMA gene promoter. This promoter region contains TGF-β-responsive Smad3-binding elements. Smad3 has been proposed to be a critical timer/delayer of development of EMT and to differentially affect various components of EMT (Masszi *et al.*, 2010; Masszi and Kapus, 2011). It will thus be of interest to determine the impact of cAMP, Epac and PKA on these recently recognized regulators of EMT. Of note, Smad3 and the regulatory subunits of PKA have been shown to coimmunoprecipitate and cAMP via PKA inhibits SMAD-mediated transcription (Schiller *et al.*, 2003; Yang *et al.*, 2008).

The ability of profibrotic stimuli to inhibit expression of Epac likely contributes to cardiac fibrosis because overexpression of Epac blocks profibrotic response and Epac expression decreases at sites that have fibrotic activity (Figure 5; Yokoyama *et al.*, 2008). The mechanisms that regulate Epac expression and inhibition of its expression by profibrotic stimuli remain to be determined; little is known regarding the transcriptional and post-transcriptional regulation of Epac expression (Basoni *et al.*, 2005; Brown *et al.*, 2011). Epac also has other roles, including altering cell migration, that may relate to its actions in fibrosis (Yokoyama *et al.*, 2008;

Grandoch *et al.*, 2010; Breckler *et al.*, 2011; Mironov *et al.*, 2011; Stokman *et al.*, 2011). siRNA-mediated inhibition of Epac1 (but not Epac2) in rat cardiac fibroblasts and its downstream mediator Rap-1 decreases cAMP-induced migration of cardiac fibroblasts, implying a role for Epac1 signalling via Rap-1 in the antifibrotic actions of cAMP (Yokoyama *et al.*, 2008). In contrast, Rap1-targeted siRNA did not affect Epac-induced inhibition of collagen and DNA synthesis, thus suggesting a role for other downstream mediators of Epac1 and requires further investigation.

The ability of cAMP acting via Epac to exert antifibrotic effects and of profibrotic agents to inhibit Epac expression suggests that strategies to increase Epac expression and activity may have therapeutic potential in preventing or treating tissue fibrosis. However, since Epac regulates numerous effects of cAMP (Gloerich and Bos, 2010; Grandoch *et al.*, 2010; Breckler *et al.*, 2011), it may be difficult to selectively alter Epac expression and actions in fibroblasts. This problem is heightened by the lack of pharmacological inhibitors of Epac and the difficulty in achieving fibroblast-specific targeting of genes (Liu *et al.*, 2010; Österreicher *et al.*, 2011).

Might other approaches be used to increase cAMP and activate Epac in fibroblasts? One possibility is to take advantage of fibroblast-selective expression of signalling components involved in regulating cAMP levels, for example, by activating GPCRs that increase cAMP formation, inhibiting GPCRs that decrease cAMP formation or inhibiting PDEs that hydrolyse cAMP. Results showing that an AC6 adenovirus or fibroblast-targeted AC6 enhances GPCR-promoted cAMP formation and antifibrotic activity (Swaney *et al.*, 2005; Liu *et al.*, 2010) imply that efforts to enhance cAMP levels in fibroblasts may have antifibrotic effects *in vivo*. Preliminary data indicate that fibroblasts selectively express certain GPCRs that regulate cAMP formation (Insel *et al.*, 2012a; the 2009 *BJP Guide to Receptors and Channels*, which is available for free download at http://www3.interscience.wiley.com/journal/122684220/issue) and particular isoforms of cAMP–PDE (data not shown) that might be targeted as ways to prevent, blunt or perhaps even reverse fibrosis. Previous data have shown that targeting cAMP-PDEs, in particular PDE4 and PDE1, by family-specific inhibitors and siRNA can inhibit fibroblast activation, EMT and collagen synthesis (Dunkern *et al.*, 2007; Kolosionek *et al.*, 2009; Sachs *et al.*, 2007; Miller *et al.*, 2011). Approaches that increase cAMP levels, such as GPCRs and/or PDE inhibitors in fibroblasts, perhaps combined with Epac or PKA activators, may be novel strategies for the treatment of tissue fibrosis (Leask, 2010; Kisseleva and Brenner, 2011). The GPCR/cAMP/Epac pathway offers intriguing possibilities for new pharmacological approaches directed at achieving antifibrosis *in vivo*.

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Conflict of interest

None.

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