

# Angiotensin-II Mediates Nonmuscle Myosin II Activation and Expression and Contributes to Human Keloid Disease Progression

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Aberrant fibroblast migration in response to fibrogenic peptides plays a significant role in keloid pathogenesis. Angiotensin II (Ang II) is an octapeptide hormone recently implicated as a mediator of organ fibrosis and cutaneous repair. Ang II promotes cell migration but its role in keloid fibroblast phenotypic behavior has not been studied. We investigated Ang II signaling in keloid fibroblast behavior as a potential mechanism of disease. Primary human keloid fibroblasts were stimulated to migrate in the presence of Ang II and Ang II receptor 1 (AT<sub>1</sub>), Ang II receptor 2 (AT<sub>2</sub>) or nonmuscle myosin II (NMM II) antagonists. Keloid and the surrounding normal dermis were immunostained for NMM IIA, NMM IIB, AT<sub>2</sub> and AT<sub>1</sub> expression. Primary human keloid fibroblasts were stimulated to migrate with Ang II and the increased migration was inhibited by the AT<sub>1</sub> antagonist EMD66684, but not the AT<sub>2</sub> antagonist PD123319. Inhibition of the promigratory motor protein NMM II by addition of the specific NMM II antagonist blebbistatin inhibited Ang II-stimulated migration. Ang II stimulation of NMM II protein expression was prevented by AT<sub>1</sub> blockade but not by AT<sub>2</sub> antagonists. Immunostaining demonstrated increased NMM IIA, NMM IIB and AT<sub>1</sub> expression in keloid fibroblasts compared with scant staining in normal surrounding dermis. AT<sub>2</sub> immunostaining was absent in keloid and normal human dermal fibroblasts. These results indicate that Ang II mediates keloid fibroblast migration and possibly pathogenesis through AT<sub>1</sub> activation and up-regulation of NMM II.

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## INTRODUCTION

Keloids are benign dermal fibroproliferative tumors (1). The *sine qua non* of keloids is the growth of the lesion beyond original wound margins (2–4). Keloids consist of a quiescent central area surrounded by an active leading edge that is frequently erythematous and pruritic (1). Histologically, keloids are seen to invade adjacent reticular dermis beneath normal-appearing papillary dermis and epidermis (2). Previous research has demonstrated that keloid fibroblasts proliferate and migrate more rapidly than control dermal fibroblasts (5–9). De-

spite these previous investigations, no clear molecular mechanisms for keloid development have been defined, and effective treatment options remain marginally effective (10). To develop a preventative therapy for this prevalent disease, it is necessary to further understand the cellular and molecular processes that cause keloid fibroblast migration and proliferation.

Angiotensin II (Ang II) is a vasoactive hormone that is best known for playing a role in cardiovascular homeostasis through the systemic renin-angiotensin system (RAS) (11,12). Ang II also acts lo-

cally to promote tissue repair in the vasculature, heart, brain, lung, kidney, liver and skin (13–16). In mammalian cells, Ang II signals through two principal receptors, angiotensin receptor 1 (AT<sub>1</sub>) and angiotensin receptor 2 (AT<sub>2</sub>) (12). AT<sub>1</sub> exerts most of the known actions of Ang II (17). In select instances, AT<sub>2</sub> activation counteracts AT<sub>1</sub>-mediated effects leading to inhibition of cell growth and promotion of apoptosis (12,18). Regulation of receptor expression is one mechanism that can determine end-organ responsiveness to Ang II (12). Ang II is implicated as a critical mediator of cutaneous wound healing and pathologic scarring (14). Ang II stimulates proliferation, migration and matrix production of human dermal fibroblasts (19–22). Immunohistochemical staining of normal skin, wounded skin and hypertrophic scars has revealed that AT<sub>1</sub> and

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AT<sub>2</sub> expression are increased in scar epithelium and endothelium and that angiotensin-converting enzymes (ACE) activity is also increased, but reports of these findings do not describe immunostaining of the deep dermis (15,16). Administration of Ang II and its nonhypertensive analog angiotensin (1–7) accelerates dermal repair in rodents (23,24). The role of Ang II signaling in keloid pathogenesis is unknown.

Nonmuscle myosin II (NMM II) is the principal motor protein in fibroblasts that is the final common effector protein of multiple promigratory signaling pathways (25–28). There are three mammalian isoforms of NMM II heavy chains, IIA, IIB and IIC. IIA and IIB are most important for migration (26). IIA and IIB also play a pivotal role in maintaining health, because mutations that affect these isoforms cause cleft lip and palate, hearing disorder, and cardiac disease (26,29,30). The role of NMM II in keloid pathogenesis is unknown. Collagen, increased levels of growth factors and cytokines, increased proliferation and increased migration have been suggested to play roles in keloid pathogenesis (1,5–9). The existence of Ang II receptors in normal and keloid fibroblasts suggests that Ang II has a role in pathogenesis of keloids. Ang II is known to increase cell migration, collagen production, proliferation and growth factors and cytokines in cardiac, renal and vascular disease (31). The role of Ang II in keloid pathogenicity is unknown. In the investigation we report, we hypothesized a role for Ang II in keloid pathogenesis. Our results showed an increased AT<sub>1</sub> expression in keloid tissues. We found that Ang II increases keloid fibroblast migration in an AT<sub>1</sub>-dependent manner by increasing NMM II expression. We demonstrated that the increased migration by Ang II was suppressed by AT<sub>1</sub> inhibitors and not AT<sub>2</sub> inhibitors. Ang II was also found to increase proliferation in an AT<sub>1</sub>-dependent manner. Ang II was found not to increase transforming growth factor (TGF)- $\beta$  or collagen I production.

**Table 1.** Origin of keloid sample.

Specimen	Age, y	Sex	Race	Location	Inciting Trauma	Treatment
1	17	Female	Black	Left ear	Piercing	None
2	17	Male	Unknown	Left ear	Piercing	None
3	28	Male	White	Left ear	Piercing	E/SI <sup>a,b</sup>
4	37	Male	Black	Left ear	Piercing	R/SI/E <sup>b</sup>
5	63	Male	White	Back	Unknown	None
6	19	Female	Black	Sternum	Thymectomy	SI/TS
7	37	Female	Black	Mandible	Unknown	E/SI <sup>b</sup>
8	26	Male	Black	Face	Shaving	SI
9	25	Female	Black	Shoulder	Unknown	None
10	14	Male	Black	Left ear	Piercing	None
11	21	Female	Black	Right ear	Piercing	None

<sup>a</sup>E, excision; R, radiation; SI, steroid injection; TS, topical steroid.

<sup>b</sup>Previously excised lesions grew back and were re-excised in this study.

## MATERIALS AND METHODS

### Chemicals

The chemicals used were Ang II, ( $\pm$ )-blebbistatin (Calbiochem, San Diego, CA, USA), PD123319, losartan (Sigma Aldrich, St. Louis, MI, USA), and EMD66684 (Tocris Bioscience, Ellisville, MO, USA) (32).

### Cell Culture

Human fibroblasts were obtained from surgical specimens per a protocol approved by the institutional review board. Normal tissues were easily distinguished, divided and explanted separately from keloid tissue. Four keloid-derived dermal fibroblast cultures, derived from the periphery of keloids from separate patient biopsy tissue samples (patients 1, 2, 10 and 11 in Table 1), were used in the study. Surgical specimens of keloids were washed, minced, and incubated in Dulbecco's Modified Eagle's medium (DMEM; Sigma Aldrich) containing 0.75% collagenase type I (Worthington Biochemical, Freehold, NJ, USA) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) at 37°C overnight. The tissue was rinsed and cells cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma Aldrich) and 1% penicillin/streptomycin at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. Keloid fibroblasts from the third to sixth passages were used.

### Cell Migration Assay

Polyethylene terephthalate-etched polycarbonate cell migration inserts (8- $\mu$ m pores; BD Falcon) were coated with 10  $\mu$ g/mL type 1 collagen (BD Biosciences, San Jose, CA, USA) and allowed to air dry. Inserts were placed over a 24-well chamber containing Ang II and/or chemical antagonists in DMEM. We added  $2 \times 10^4$  cells in DMEM to the insert. The chamber was placed in an incubator for 6 h, after which the upper surface of the insert was swabbed to remove nonmigratory cells. Migrated cells were fixed and stained with a Protocol HEMA-3 cell staining kit (Fisher, Waltham, MA, USA). Migration was quantified by counting the number of cells in 5 random fields at a 40 $\times$  objective under a light microscope.

### Proliferation Assay

Proliferation was assessed using a CellTiter 96<sup>®</sup> AQ<sub>ueous</sub> Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI, USA). Cells were plated at  $2 \times 10^3$  cells/well (200  $\mu$ L) on a 96-well plate in DMEM with 10% FBS for 24 h at 37°C and 5% CO<sub>2</sub>. After 48 h under normal conditions cells were washed with phosphate-buffered saline, and serum starved in 1% FBS overnight. Following serum starvation, treatment conditions were added and cells were incubated for 24 h at 37°C and 5% CO<sub>2</sub>. Following 24-h treatment with experimental conditions, 20  $\mu$ L of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-

2H-tetrazolium (MTS), inner salt (Promega Corporation; Madison, WI, USA) was added to each well. The plate was then incubated at 37°C and 5% CO<sub>2</sub> and the absorbance at 490-nm wavelength was recorded.

**RNA Extraction and Analysis**

Total RNA was extracted from fibroblasts at 6 h, following Ang II stimulation, by using the Qiagen RNeasy Universal Plus Mini Kit (Valencia, CA, USA) according to the manufacturer’s instruction manual. All RNA samples were treated with RNase-free DNase 1 to remove potential contaminating genomic DNA. Total RNA (10 ng) was reverse transcribed to cDNA and then amplified with the Qiagen Quantitect SYBR Green RT-PCR kit. All reagents, primers and probes were purchased from Qiagen. Each sample was assayed in triplicate on the Stratagene Mx3005P qPCR system (Santa Clara, CA, USA). Results were analyzed by using MxPro software (Agilent Technologies Inc., Santa Clara, CA, USA). The housekeeping gene S9 was used to normalize mRNA concentration.

**Western Blot Analysis**

Keloid fibroblasts were seeded on 6-well plates (5 × 10<sup>4</sup> cells/well) and serum starved overnight and stimulated. Protein was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (NuPAGE 4%–12% Bis-Tris gels; Invitrogen) and transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA, USA). The membranes were then probed with anti-NMM IIA (1:1000 dilution; rabbit polyclonal, ab24762; Abcam, Cambridge, MA, USA), anti-NMM IIB (1:1000 dilution; goat polyclonal N17, sc-47205; Santa Cruz Biotechnology), anti-AT<sub>1</sub> (1 µg/mL rabbit polyclonal, N-10, sc-1173; Santa Cruz Biotechnology) and anti-AT<sub>2</sub> (1 µg/mL H-143, rabbit polyclonal sc-9040; Santa Cruz Biotechnology) and anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (1:200 dilution; mouse monoclonal 2D4A7, sc-59541; Santa Cruz Biotechnology) antibodies in Near Infra-

Red Blocking Buffer overnight. Membranes were then incubated in secondary antibody: donkey anti-rabbit IRDye700, 611-730-127; donkey anti-goat IRDye800, 605-732-125; donkey anti-mouse IRDye 800, 610-732-124 (1:20,000 dilution; Rockland Immunochemicals, Gilbertsville, PA, USA). The membrane was scanned by using an Odyssey Infrared Imager (Model 9120, Li-Cor Inc, Lincoln, NE, USA), and band intensity was quantified with Odyssey software.

**Immunohistochemistry**

All human studies were approved by the Duke University Medical Center (DUMC) Institutional Review Board. Specimens were obtained for immunohistochemical staining from the DUMC Department of Pathology repository. Sections (5 µm) were mounted on silanized charged slides. After deparaffinization, slides were covered with 3% hydrogen peroxide and placed in retrieval buffer for 20 min at 80°C, followed by washing in tris-buffered saline containing 0.5% Tween-20 (TBS-T: 0.5 mol/L Tris Base, 9% NaCl, 0.5% Tween 20, pH 8.4). Slides were incubated with primary anti-NMM IIA (1:100 dilution; rabbit polyclonal, ab24762; Abcam), anti-NMM IIB (1:100 dilution; rabbit polyclonal N17, sc-47205; Santa Cruz Biotechnology), anti-AT<sub>1</sub> (rabbit polyclonal, N-10), and anti-AT<sub>2</sub> (rabbit polyclonal, H-143) (sc-1173, sc-9046, respectively; Santa Cruz Biotechnology). Slides were incubated for 45 min with biotinylated secondary antibodies (1:200 dilution; goat anti-rabbit IgG; Vector, Burlingame, CA, USA). After washing in TBS-T, the slides were incubated with the detection system (Vectastain Elite ABC, Vector). Tissue staining was visualized with a DAB substrate chromogen solution (Innovex, Richmond, CA, USA). Slides were counterstained with hematoxylin. To quantify the degree of positive staining for AT<sub>1</sub> and AT<sub>2</sub>, each slide was subjected to semiquantitative analysis by using the following system: 0, 1 (0%–25% reactivity), 2 (35%–50% reactivity), 3 (50%–75% reactivity) and 4 (75%–100% reactivity).

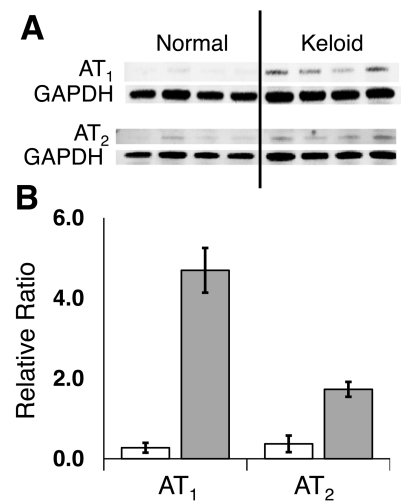
**Statistical Analysis**

All quantitative data are presented as the mean and standard error of the mean of three independent experiments, performed in triplicate for each condition, using each of the four cell lines. Statistical analysis was performed by using either analysis of variance or two-sided Student *t* test where appropriate. Difference was considered significant at *P* ≤ 0.05.

**RESULTS**

**Angiotensin II Induces Migration of Keloid-Derived Dermal Fibroblasts in a Dose-Dependent Manner via AT<sub>1</sub> and NMM II Activation**

To investigate the expression of AT<sub>1</sub> and AT<sub>2</sub> expression in keloid fibroblasts, fibroblasts were explanted from the periphery of keloids from four separate patient biopsies. AT<sub>1</sub> and AT<sub>2</sub> expression was significantly increased compared with normal fibroblasts as determined by Western



**Figure 1.** Angiotensin II receptor 1 (AT<sub>1</sub>) and receptor 2 (AT<sub>2</sub>) in fibroblasts explanted for keloid and normal tissue. (A) Western blot of AT<sub>1</sub> and AT<sub>2</sub> expression in four fibroblast cell lines from keloid and normal tissue. (B) Quantification of Western blotting showed that AT<sub>1</sub> and AT<sub>2</sub> expression was significantly increased in keloid fibroblasts (gray columns) compared with normal fibroblasts (white columns).

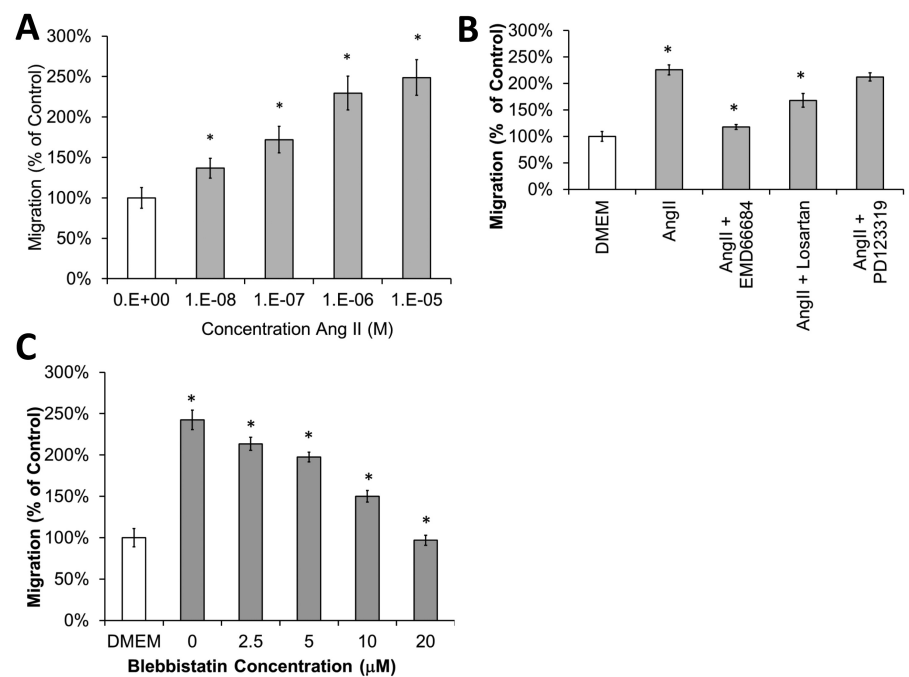
blotting ( $P < 0.05$ ; Figure 1). The relative expression in keloid fibroblasts of AT<sub>1</sub> was approximately 2.7 times greater than AT<sub>2</sub>.

We examined the effect of Ang II on keloid fibroblast migration in a modified Boyden chamber migration assay. Ang II demonstrated a dose-dependent induction of fibroblast migration, with a maximum increase of 2.6-fold at 10<sup>-5</sup> mol/L compared with controls ( $P \leq 0.05$ ; Figure 2A). Because no significant difference in keloid fibroblast migration was observed between 10<sup>-6</sup> mol/L and 10<sup>-5</sup> mol/L Ang II, 10<sup>-6</sup> mol/L of Ang II was employed in subsequent migration assays. To elucidate whether Ang II-induced keloid fibroblast migration through AT<sub>1</sub> or AT<sub>2</sub>, the AT<sub>1</sub>-selective antagonists EMD66684 (10<sup>-5</sup> mol/L) and losartan (10<sup>-6</sup> mol/L), and AT<sub>2</sub>-selective antagonist PD123319 (10<sup>-5</sup> mol/L) were used.

EMD66684 and losartan inhibited Ang II-induced keloid fibroblast migration by 108% and 58%, respectively ( $P \leq 0.05$ ; Figure 2B), but PD123319 (10<sup>-5</sup> mol/L) failed to significantly inhibit migration. We investigated the role of NMM II ATPase activity in Ang II-induced keloid fibroblast migration by using the NMM II inhibitor blebbistatin. Ang II-induced keloid fibroblast migration was inhibited by blebbistatin in a dose-dependent manner (Figure 2C). Blebbistatin inhibited Ang II-induced keloid fibroblast migration by 62% at 10  $\mu$ mol/L blebbistatin, with complete inhibition at 20  $\mu$ mol/L.

### Angiotensin II Induces the Expression of NMM II Isoforms A and B in a Dose-Dependent Manner via AT<sub>1</sub>.

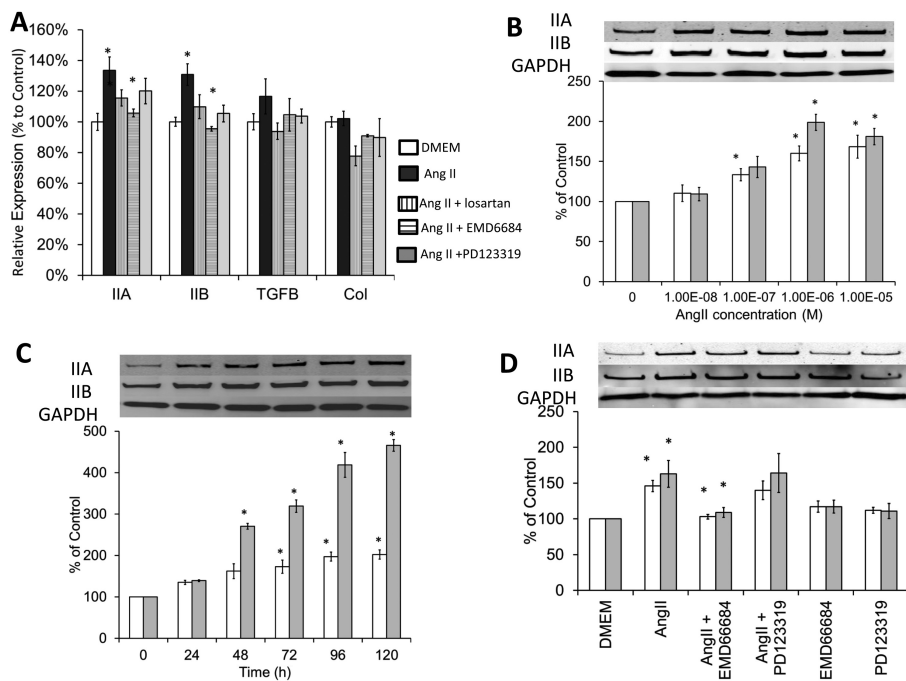
We sought to investigate how Ang II affected IIA and IIB expression in keloid fibroblasts. We compared the transcriptional response of keloid fibroblasts following 6 h 10<sup>-5</sup> mol/L Ang II stimulation. IIA and IIB mRNA expression was increased to 134% and 131% ( $P \leq 0.05$ , Figure 3A). To investigate if this increase was AT<sub>1</sub> or AT<sub>2</sub> dependent, keloid fibroblasts were incubated with Ang II in the presence of AT<sub>1</sub> antagonists, losartan or EMD66684 or the AT<sub>2</sub> antagonist PD123319. Both AT<sub>1</sub> antagonists de-



**Figure 2.** Ang II-induced migration of keloid fibroblasts (A) Ang II induces keloid fibroblast migration in a dose-dependent manner. Ang II (10<sup>-5</sup> to 10<sup>-8</sup> mol/L) was added to the medium, and keloid fibroblast migration was assessed by a Boyden chamber assay. (B) Ang II-induced migration of keloid fibroblasts is mediated by the AT<sub>1</sub>. EMD66684 and losartan (AT<sub>1</sub> antagonists) or PD123319 (AT<sub>2</sub> antagonist) was added to the medium at a concentration of 10<sup>-5</sup> mol/L and keloid fibroblast migration assessed in response to 10<sup>-6</sup> mol/L Ang II. EMD66684 and losartan, but not PD123319, inhibited Ang II-induced keloid fibroblast migration. (C) Effects of the nonmuscle myosin II inhibitor blebbistatin on Ang II-induced keloid fibroblast migration. Blebbistatin (0–20  $\mu$ mol/L) was added to the medium, after which Ang II-induced migration was assessed by a Boyden chamber assay. Blebbistatin inhibition of Ang II-induced keloid fibroblast migration was dose dependent. All data are presented as percentage of migration in the absence of Ang II, mean  $\pm$  SEM; n = 9; \* $P \leq 0.05$ .

creased Ang II-stimulated IIA and IIB expression; losartan decreased IIA Ang II-stimulated expression from 134% to 116% ( $P = 0.14$ ). EMD6684 decreased Ang II-stimulated IIA expression to 106% ( $P = 0.05$ ). Losartan decreased IIB Ang II-stimulated expression from 131% to 110% ( $P = 0.07$ ). EMD6684 decreased Ang II-stimulated IIB expression to 95% ( $P = 0.01$ ). PD123319 did not decrease Ang II-stimulated IIA or IIB expression significantly ( $P \leq 0.05$ ). Both TGF- $\beta$  and collagen have been suggested to play a role in keloid pathogenicity. We found that Ang II did not significantly increase TGF- $\beta$  or collagen mRNA expression ( $P \leq 0.05$ ). Following 24-h stimulation with 10<sup>-6</sup> mol/L Ang II, IIA and IIB protein expression were increased when compared

with controls ( $P \leq 0.05$ ; Figure 3B). A dose of 10<sup>-7</sup> mol/L Ang II was the lowest dose that significantly increased IIA and IIB expression (Figure 3B). IIA and IIB expression increased 1.6-fold and 1.9-fold, respectively, at 10<sup>-6</sup> mol/L Ang II compared with controls ( $P \leq 0.05$ ; Figure 3C). Higher doses of Ang II (10<sup>-5</sup> mol/L) did not significantly increase NMM II expression, compared with 10<sup>-6</sup> mol/L of Ang II. Therefore, 10<sup>-6</sup> mol/L Ang II was used to evaluate the effect of AT<sub>1</sub> and AT<sub>2</sub> antagonists (10<sup>-5</sup> mol/L) on the expression of IIA and IIB (Figure 3D). Expression of both NMM II isoforms continued to increase temporally with Ang II stimulation up to 120 h (Figure 3C). Ang II-induced increases in IIA and IIB expression was completely inhibited by the



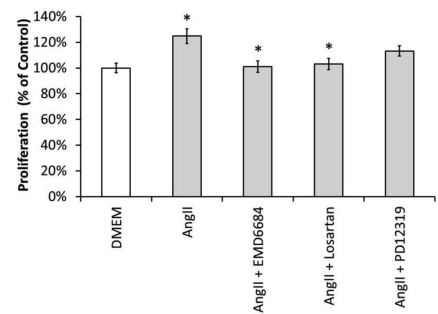
**Figure 3.** Ang II-Induced nonmuscle myosin II expression in keloid fibroblasts. (A) Keloid fibroblasts were serum starved overnight, followed by 6 h of stimulation. Total RNA was harvested at indicated conditions and subjected to quantitative real-time PCR analysis. The expression of each gene was normalized to the expression of housekeeping gene S9 and reported as percent expression relative to the control of DMEM alone. A representative Western blot is shown above the mean  $\pm$  SEM of three experiments in separate primary cell lines. (B) Ang II induces dose-dependent expression of NMM IIA (white columns) and IIB (gray columns). Cells were incubated in Ang II ( $10^{-5}$  to  $10^{-8}$  mol/L) for 24 h prior to lysate collection. (C) Ang II-induced expression of NMM IIA and IIB is time dependent. Cells were stimulated with  $10^{-6}$  mol/L Ang II for 24–120 h, and lysates were collected. NMM IIA was increased two-fold (white columns) and IIB more than four-fold (gray columns). (D) Ang II-induced expression of NMM IIA and IIB in keloid fibroblasts is mediated by  $AT_1$ . EMD66684 ( $AT_1$  antagonist) or PD123319 ( $AT_2$  antagonist) was added to the medium at a concentration of  $10^{-5}$  mol/L and cells were stimulated with  $10^{-6}$  mol/L Ang II for 24 h. EMD66684, but not PD123319, inhibited Ang II-induced NMM IIA (white columns) and IIB expression (gray columns). \* $P \leq 0.05$ .

$AT_1$  antagonist EMD66684, but not the  $AT_2$  antagonist PD123319 ( $P \leq 0.05$ ; Figure 3D).

**Ang II Increased Keloid Fibroblast Proliferation in an  $AT_1$ -Dependent Mechanism.**

Keloid disease is characterized by persistent growth of the wound tissue after reepithelialization and extension of scar tissue beyond the original borders of the wound. To investigate if Ang II increased proliferation of keloid fibroblasts, the MTS assay was used to demonstrate increase proliferation. Following 18 h of

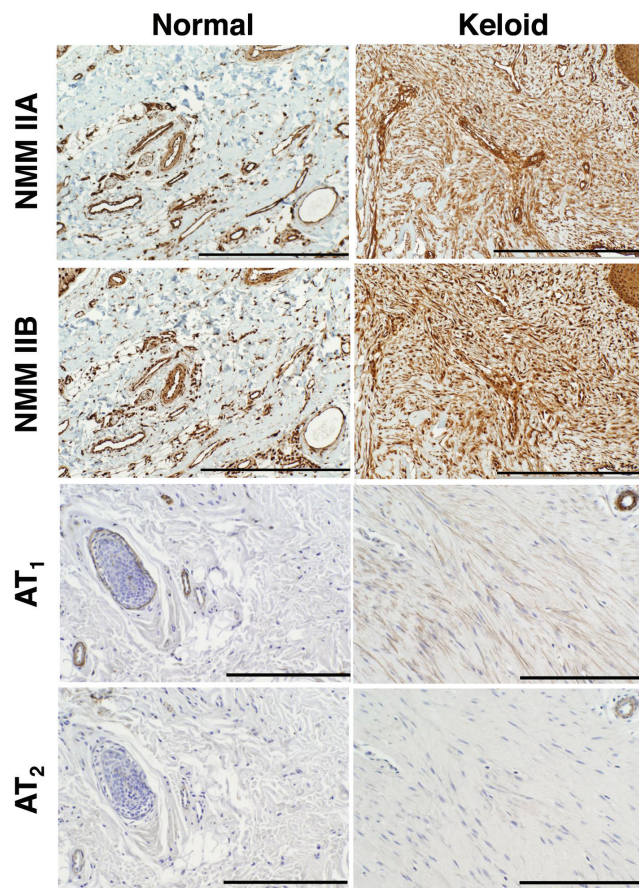
serum starving, keloid fibroblast proliferation was stimulated with Ang II at concentrations from 0 to  $10^{-5}$  mol/L; the maximum increase in proliferation was observed at 1  $\mu$ mol/L (data not shown). To determine if this observed increase in Ang II stimulated proliferation, we cultured keloid fibroblasts with 0– $10^{-5}$  mol/L losartan, EMD6684 or PD123319 in the presence of  $10^{-6}$  mol/L Ang II. The  $AT_1$  antagonists were shown to decrease the Ang II-stimulated increase in proliferation significantly. PD123319, the  $AT_2$  antagonist, did not significantly decrease the Ang II-stimulated proliferation (Figure 4).



**Figure 4.** Ang II-induced proliferation of keloid fibroblasts. Keloid fibroblasts were serum starved overnight, followed by 24-h stimulation with Ang II ( $10^{-6}$  mol/L) at the indicated conditions. The MTS assay was used to determine proliferation. Ang II significantly increased proliferation.  $AT_1$  antagonists decreased Ang II-stimulated proliferation, whereas  $AT_2$  antagonist did not. \* $P \leq 0.05$

**NMM II A and II B expression Is Increased at the Active, Immature Margins of Keloids**

Eleven keloid specimens with surrounding normal skin were selected for immunohistochemical staining for IIA and IIB. Demographic and clinic data are listed in Table 1. Representative histologic sections of IIA and IIB immunolocalization in keloid tissue at 10 $\times$  objective are given in Figure 5. The staining patterns were not significantly different between the two proteins, and the description below applies to both IIA and IIB immunolocalization. The normal, nonkeloidal region (Figure 5A, B) demonstrates sparse fibroblasts and an increased amount of positive-staining vasculature. Staining for NMM II in the region corresponding to the active, immature leading edges of the keloid reveals a densely fibroblast-populated hypercellular region with significantly increased NMM II expression (Figure 5C, D). NMM II expression is robust in the cytoplasm relative to fibroblasts in nonkeloidal regions. Semi-quantitative analysis of the expression is given in Table 2 and shows that keloid regions express greater NMM than nonkeloid regions.



**Figure 5.** Representative immunohistological staining of NMM IIA and NMM IIB and AT<sub>1</sub> and AT<sub>2</sub>. (B, C) NMM IIA and IIB immunostaining is increased in the periphery of keloids. This is a hypercellular region of dermis densely populated by fibroblasts, with significantly increased NMM IIA and IIB expression in the cytoplasm and perinuclear regions of the dermal fibroblasts. (E, F) In normal, nonkeloidal skin, no staining of dermal fibroblasts was observed for either AT<sub>1</sub> or AT<sub>2</sub>. (G) Staining for AT<sub>1</sub> in keloid revealed a heterogeneous distribution of expression within dermal fibroblasts, with the lowest signal observed in regions of fibroblast hypocellularity and highest signal observed in regions of relative fibroblast hypercellularity. (H) No staining for AT<sub>2</sub> was observed in dermal fibroblasts of keloids. NMM IIA, NMMIIB, AT<sub>1</sub> and AT<sub>2</sub> scale bars = 500  $\mu$ m.

#### Expression of AT<sub>1</sub> Is Increased in Dermal Fibroblasts at the Peripheral, Hypercellular Regions of Keloids

AT<sub>1</sub> was present in the vascular endothelial, myoepithelial cells of eccrine sweat glands, and smooth muscle cells of normal skin surrounding the excisional margins of keloid specimens, but absent in dermal fibroblasts (Figure 5E). AT<sub>2</sub> had a similar pattern of staining (Figure 5F). In keloids, AT<sub>1</sub> distribution was heterogeneous, demonstrating regional variation in dermal fibroblast staining. The highest levels of staining for AT<sub>1</sub> were observed

in the relatively hypercellular region of the keloid located more peripherally within the reticular dermis. Staining for AT<sub>1</sub> was also observed in fibroblasts within the more hypocellular central region of the keloid that contained thick hyalinized keloidal collagen (Figure 5G). Staining in the central hypocellular region was less intense than in the peripheral hypercellular region. AT<sub>2</sub> was absent throughout the keloid dermis (Figure 5H). The distribution of staining was consistent in 10 of the 11 keloid specimens, irrespective of treatment. In the single

**Table 2.** Semiquantitative analysis of AT<sub>1</sub> and AT<sub>2</sub> expression in 11 keloid specimens;  $P < 0.05$ .

Score range	Number of specimens	
	AT <sub>1</sub>	AT <sub>2</sub>
1%–25%	2	9
25%–50%	4	2
50%–75%	5	0
75%–100%	0	0

specimen that failed to demonstrate staining of dermal fibroblasts for either AT<sub>1</sub> or AT<sub>2</sub>, a pattern of keloidal collagen distribution characteristic of a mature, quiescent keloid lacking the hypercellular foci of fibroblasts identified in the other specimens was observed.

#### DISCUSSION

Keloids are characterized by the advancement of fibroproliferative tissue beyond the margins of the original wound. It is hypothesized that the underlying mechanism of this expansion is an abnormal response of keloid-derived fibroblasts to extracellular stimuli. Ang II has been shown to have an important role in both normal and pathologic wound healing responses. In this study, we tested the hypothesis that Ang II contributes to keloid pathogenesis by inducing keloid fibroblast migration through activation of AT<sub>1</sub>-dependent signaling and NMM II upregulation and activation. The results of this study demonstrate that Ang II stimulates NMM II-dependent migration, proliferation and IIA and IIB expression in keloid fibroblasts *in vitro* through AT<sub>1</sub>-dependent, but not AT<sub>2</sub>-dependent, signaling pathways. We demonstrate that TGF- $\beta$  and collagen, thought to have a role in pathogenesis of keloids, do not increase in response to Ang II. In addition, *in vivo* expression of AT<sub>1</sub>, IIA and IIB is upregulated in keloid scars, with the highest levels of expression in dermal fibroblasts positioned within the leading edge of fibroproliferation found at the keloid margins. A low level of expression of AT<sub>2</sub> in dermal fibroblasts was observed in Western blot analysis and immunohistochemical staining.

AT<sub>1</sub> is ubiquitously and abundantly distributed in adult tissues, including blood vessel, heart, kidney, adrenal gland, liver, brain and lung. In adults, expression of the AT<sub>2</sub> receptor under normal conditions is largely restricted to the adrenals, kidneys, uterus, ovary, heart and specialized nuclei in the brain (33). We present data showing that AT<sub>1</sub> expression is greater than AT<sub>2</sub> expression in keloid tissue and in fibroblasts isolated from keloids. It is possible that this difference in AT<sub>1</sub> and AT<sub>2</sub> expression is a cause of keloid disease. It is not known why this change in AT<sub>1</sub> and AT<sub>2</sub> expression occurs. Multiple factors such as hormones, cytokines, and metabolic and growth factors, could be involved in determination of the AT<sub>1</sub>:AT<sub>2</sub> ratio on cells (34,35–37).

Previous studies have demonstrated the role of Ang II in many aspects of wound repair (38). Exogenous administration of up to 100 µg Ang II per day improved diabetic wound healing in mice, and the Ang II analog (1–7) also improved wound healing (23,24). In addition, studies have also demonstrated increased endogenous levels of Ang II, ACE activity and angiotensin receptors in dermal wound repair (39,40). Most recently, Yahata *et al.* reported that the major determinant of accelerated cutaneous wound repair by Ang II is increased fibroblast migration, an effect mediated by AT<sub>1</sub> signaling (22). Yahata *et al.* employed both normal human dermal fibroblasts *in vitro* and AT<sub>1</sub> knock-out mice to demonstrate the role of Ang II in the normal cutaneous wound healing response. The demonstration of the role of Ang II in wound healing using an AT<sub>1</sub> knock-out mouse provided some of the first evidence supporting a role *in vivo* for endogenous Ang II signaling during wound repair.

Although keloid-derived fibroblasts have been shown to possess increased migratory activity when compared with fibroblasts isolated from normal human dermis, migratory activity in keloid fibroblasts in response to stimulation with Ang II has not been reported. Similar to the findings of Yahata *et al.* that Ang II increases cell migration, we found that

Ang II stimulates keloid fibroblast migration through an AT<sub>1</sub>-dependent and an AT<sub>2</sub>-independent mechanism (22). This increase in cell migration could be caused by upregulation of NMM II, because we observed that Ang II increased NMM expression in an AT<sub>1</sub> dependent mechanism. We also found that Ang II stimulation of AT<sub>1</sub> signaling promoted proliferation in keloid fibroblasts but did not increase TGF-β or collagen expression. The implication of these findings is that inhibition of AT<sub>1</sub> signaling by the sartan class of drugs may be useful to prevent keloid disease.

The sartans are Ang II receptor antagonists that were introduced more than 10 years ago (41). They have a highly selective affinity for the AT<sub>1</sub> receptor, with IC<sub>50</sub> values in the range of 1 to 2 nmol/L; and an affinity for AT<sub>1</sub> usually 10,000 to 20,000 times higher than that for AT<sub>2</sub>. One of the more commonly used sartans, losartan, is dosed per oral at 50–100 mg/day (42). The sartans are safe with minimal side effects (43). Exceedingly rare cutaneous side effects have been reported, including pityriasis rosea and mucocutaneous bullous pemphigoid, but these case reports should not discourage the use of sartans because the risk/benefit ratio is practically so small (44,45). EMD66684 is a highly potent and very selective nonpeptide angiotensin AT<sub>1</sub> receptor antagonist (IC<sub>50</sub> values are 0.7 and >10,000 nmol/L for AT<sub>1</sub> and AT<sub>2</sub> receptors, respectively). Both ATR1 inhibitors evaluated in this study decreased Ang II-stimulated migration and could potentially be used to treat keloids.

The NMM II isoforms are the principal force-generating myosins in fibroblasts (25). NMM II has emerged as the master regulator and integrator of cell migration, mediating each of the component processes of migration (28,30). Given this role in fibroblast dynamics, our demonstration that Ang II-induced keloid fibroblast migration is NMM II dependent is not surprising. More significant, however, is our demonstration that Ang II increases the expression of IIA and IIB as a function of both dose and time. This is

the first time that Ang II has been shown to increase NMM II expression. Furthermore, Ang II was found to increase activation of NMM II in cell migration, as inhibition of NMM II by blebbistatin inhibited Ang II-stimulated effects. Ang II upregulation and activation of NMM II in keloid fibroblasts is a two-tiered mechanism to promote migration.

Further supporting a role for NMM II in keloid pathogenesis is the immunohistochemical evidence that the expression of AT<sub>1</sub>, IIA and IIB are increased in keloid tissue *in vivo*, and that the highest levels of expression are observed in dermal fibroblasts located at the peripheral hypercellular margins of the keloid, where migration is most active. AT<sub>2</sub> expression was also increased in keloid tissue but to a lesser extent. This is first report of increased AT<sub>1</sub> and NMM II expression *in vivo*. The dynamic nature of keloid disease is characterized clinically and histopathologically by the regression and quiescence of the central region of the keloid while the margin remains active, growing into the bordering normal skin. The fibroblasts located at this hypercellular, expanding margin drive the fibroproliferation and expansion that have become the defining elements of keloid disease. The localization of increased IIA and IIB expression within the dermal fibroblasts of the keloid margins correlates with the biological and histopathological characterization. Given the functional role of NMM II in fibroblast migration, this observation further supports the theory that increased expression and activation of NMM II in keloid fibroblasts enhances cell migration and keloid growth.

In summary, our data demonstrate that Ang II induces NMM II-dependent migration, and increased NMM II expression via AT<sub>1</sub> signaling in keloid fibroblasts. Immunostaining of keloid specimens reveals increased NMM II and AT<sub>1</sub> expression that is maximal at the active leading edge of the lesion. These results suggest a putative mechanism of keloid pathogenesis in which Ang II signaling induces keloid fibroblast migration associated with an increase in NMM

II expression and activation. Inhibition of Ang II signaling with AT<sub>1</sub> antagonists such as the class of sartans may offer a novel, effective strategy to treat keloids.

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## DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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