Regulation of proximal tubular osteopontin in experimental hydronephrosis in the rat

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Background. Osteopontin is a tubular-derived glycoprotein with macrophage chemoattractant properties. Our previous observations demonstrate that osteopontin is involved in the accumulation of macrophages within the renal cortex of rats following unilateral ureteral obstruction (UUO).

Methods. The present study performed Northern and Western blot analyses of isolated proximal tubular cells exposed to exogenous angiotensin II, and cultured rat proximal tubular cells subjected to one hour of cyclic mechanical stretch, which provided insight into mechanisms involving the proximal tubular renin-angiotensin system in the increased expression of cortical osteopontin following hydronephrosis.

Results. In situ hybridization, using a 35S-labeled antisense riboprobe, showed osteopontin mRNA transcription localized to the cortical tubules of the obstructed kidney. Freshly isolated proximal tubules incubated with angiotensin II (10^{-5} M) for one hour had increased osteopontin mRNA and protein expression by Northern and Western blot analyses, respectively. Pre-treatment of proximal tubules with losartan (10^{-5} M) for one hour prior to the addition of exogenous angiotensin II (10^{-5} M) decreased osteopontin mRNA and protein expression. Rat proximal tubule cells subjected to cyclic mechanical stretch for one hour exhibited a 2.1-fold increment in osteopontin mRNA levels, which was normalized following pre-treatment with losartan.

Conclusions. This study provides evidence that angiotensin II, produced by the proximal tubule in the obstructed kidney as a result of mechanical injury, possibly mechanical stretch, may stimulate angiotensin II type I receptor activation, leading to up-regulated osteopontin expression and secretion by the proximal tubule, thereby facilitating macrophage recruitment into the renal interstitium.

Renal cortical scarring develops as a sequela to urinary tract obstruction and is principally manifested as interstitial fibrosis. Ample clinical and experimental evidence has shown that it is the degree of interstitial fibrosis that directly correlates with the extent of renal functional impairment regardless of the disease or model studied. An initial event in progressive interstitial fibrosis is macrophage infiltration of the tubulointerstitial compartment [1]. Recent attention has focused on increased osteopontin expression by renal cortical tubular epithelium as being a forerunner to renal cortical interstitial macrophage infiltration and the development of pro-fibrogenic consequences that culminate in interstitial fibrosis in a number of experimental models [2–5].

The functional abnormalities after ureteral ligation have been well summarized by Klahr [6]. Only recently have the early cell biologic events post-ureteral ligation been studied. Our laboratory [7] and others [8, 9] have observed one such event, namely, renal interstitial macrophage infiltration, which begins to increase as early as 12 hours after ureteral obstruction and continues to rise over the course of days, thereafter. The signal for renal leukocyte recruitment immediately after ureteral ligation is macrophage-specific since there are few T-lymphocytes noted and an absence of polymorphonuclear leukocytes [10, 11].

Osteopontin is a highly acidic, negatively-charged, secreted glycosylated phosphoprotein [3]. It is also known as uropontin, early T-lymphocyte activation gene-1, secreted phosphoprotein I, and bone sialoprotein I [12]. Although it was originally isolated as a matrix molecule in bone, it is also found normally in the renal medulla in the loop of Henle and distal convoluted tubules, but is absent from normal renal cortex with the exception of the parietal epithelium of Bowman’s capsule [3, 12]. It is produced by many other epithelial cells and is found in the plasma and various body secretions including milk, urine, and bile [12].

It was the intent of this study to determine the localization and mechanism of osteopontin synthesis in diseased renal cortex. Furthermore, another goal was to discern how osteopontin expression is regulated in the proximal tubular epithelium of obstructed kidneys of rats with unilateral ureteral obstruction (UUO). We postulate that within the initial one to five hours following UUO, the proximal tubular epithelium within the obstructed kidney is...
mechanically perturbed, possibly by membrane stretch, and consequently exhibits increased angiotensin II synthesis and autocrine angiotensin II type 1 receptor stimulation, culminating in up-regulated osteopontin expression by the proximal tubular epithelium.

METHODS

Experimental animals

Male Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, MA, USA), weighing 150 to 200 g, were used in this study. Animals were fed standard rodent chow (Purina Chows, St. Louis, MO, USA) and were given water ad libitum. For proximal tubular osteopontin localization studies using in situ hybridization and immunohistochemical labeling, rats were separated into two groups: UUO and sham-operated rats (SOR). With Brevital (50 mg/kg/behavior i.p.; E. Lilly, Indianapolis, IN, USA) anesthesia, animals underwent either left proximal ureteral obstruction or a sham operation. Both the obstructed kidney and the contralateral unobstructed kidney (CUK) as well as normal kidneys from SOR were harvested from UUO animals at 12, 24, 48, and 96 hours post-ureteral ligation or a sham procedure.

Tissue preparation and osteopontin immunolocalization

For immunohistochemical labeling studies, kidneys were perfused with 0.9% NaCl via an infrarenal aortic cannula for three to five minutes to remove circulating blood cells. Kidneys were removed, sectioned coronally, immersed in methanol-Carnoy’s fixative for 24 hours, and then placed in absolute ethanol. After fixation, midcoronal sections were immersed in a solution containing 5 m NaCl, 0.05 m Tris (hydroxymethyl) aminomethane (Tris), pH 7.4, 20 × dextran sulfate solution, 0.1 mg/ml salmon sperm DNA and 1.0% sodium dodecyl sulfate (SDS), with the addition of a 2B7 cDNA probe for rat osteopontin (kindly provided by Dr. C. Giachelli, University of Washington, Seattle, WA, USA), or a human GAPDH cDNA probe (Clontec, Palo Alto, CA, USA), which yields 1.6 kb and 1.3 kb mRNA transcripts, respectively. The cDNA probe was labeled with [32P]deoxyctydine triphosphate, using a random primer cDNA labeling kit (Boehringer-Mannheim, Indianapolis, IN, USA). After hybridization at 65°C overnight, blots were washed, and quantitative densitometry was performed on the osteopontin and GAPDH autoradiograms using a computer-based measurement system. GAPDH was used as a reference probe to correct for variations in loading of RNA samples. The mRNA levels for osteopontin were expressed as ratios of the optical density of osteopontin to that of GAPDH. The peak optical density reading of each band on the autoradiograph is arbitrarily reported as densitometric units.

In situ hybridization

Riboprobe in situ hybridization was performed on 10% neutral buffered formalin-fixed paraffin sections according to established protocols [14]. 2B7, a rat osteopontin cDNA probe was linearized with BglII and Mam I to generate antisense and sense 35S-labeled (using 35S-labeled UTP; New England Nuclear, Boston, MA, USA) riboprobes with T7 and T3 polymerase, respectively. Probes were separated from unincorporated precursors using G-50 Quick Spin columns (Boehringer-Mannheim). Hybridizations were performed overnight at 50°C. Slides were washed and autoradiograms generated using Kodak BioMax film. Exposure times were five days. Controls included substituting a 35S-labeled sense probe for the antisense probe used and an incubation in 0.1 mg/ml ribonuclease (RNase) A for 90 minutes at 25°C. Epiluminescence photomicroscopy was utilized to provide histologic localization of the mRNA signal.
Proximal tubule preparation

Proximal tubules were isolated from normal rats using previously published methods [14]. Normal rat kidneys were perfused with Krebs buffered salt solution (KBSS). After clearing, each kidney was injected with 2 ml of collagenase (Worthington Chem., Type I). Renal cortex was finely minced and placed in 20 ml ice-cold KBSS and washed twice. After the second wash, 1 ml of collagenase per kidney was added and the minced kidney cortex was gassed with CO₂ at 37°C for 45 minutes with mild agitation. Every 10 minutes additional CO₂ was applied. After complete homogenization of renal cortical specimens, the mixture was strained, moralized, and centrifuged at 4°C at 6,000 rpm for one minute. The pellet was washed in ice cold KBSS twice and centrifuged for 30 seconds at 6,000 rpm. The pellet was resuspended in 50% Percoll solution and centrifuged for 30 minutes at 15,000 rpm. A glass pipette was used to remove the desired fourth band of proximal tubules. This material was subsequently centrifuged at 6,000 rpm for one minute. The pellet was washed in KBSS twice and then, resuspended in Dulbecco’s modified Eagle’s medium (DMEM)/Ham’s F12.

Western blot analysis

Specimens from freshly isolated proximal tubules (see section on proximal tubule isolation) containing approximately ~500 mg of protein, were sonicated in extraction buffer containing 10 mM Tris-HCl (pH 7.5), 1 mM ethylenediaminetetraacetic acid (EDTA), 2% SDS, and a minicomplete protease inhibitor cocktail tablet (Boehringer Mannheim) made up to a 10 ml volume. Samples for Western blot
analysis were extracted from normal rat proximal tubules, incubated in DMEM/Ham’s F12 with 10% fetal calf serum (FCS) for one hour, and normal rat proximal tubule preparations incubated in the same medium, but stimulated with $10^{-7}$ M, $10^{-6}$ M, and $10^{-5}$ M angiotensin II (Sigma). Other aliquots of the normal rat proximal tubule preparations were pre-treated with losartan ($10^{-5}$ M; Merck & Co.) prior to the one hour incubation with angiotensin II ($10^{-5}$ M).

Following extraction, the homogenates were stored at −70°C after boiling for 10 minutes. The protein concentration of the homogenates was determined with the BioRad assay. Samples containing 20 μg of protein were diluted in SDS-PAGE Laemmli buffer and loaded onto a precast gradient gel. For Western blotting, the gel was removed and placed on an immobilon (PVDF) transfer membrane (Millipore) using filter paper saturated with 300 mM Tris buffer (pH 10.4) in 5% methanol and, then covered with additional filter paper saturated with 25 mM Tris, 40 mM glycine and 20% methanol. This transfer unit was placed in a MilliBlot-SDE transfer system (Millipore) and run for 30 minutes. Transfer was judged by the standard bands (Amersham Standards) on the transfer membrane. The gel was stained in Coomassie Brilliant Blue for two hours to determine the transfer efficiency. The membrane was washed in 20 mM Tris-HCl (pH 7.6) with 137 mEq/liter NaCl buffer with 0.1% Tween-20 and 5% wt/vol nonfat dry milk for one hour at room temperature and then in a saline buffer for 15 minutes. A monoclonal mouse anti-rat osteopontin antibody (MPIIIIB10, 1:4,000 dilution) was incubated for one hour followed by a horseradish peroxidase-conjugated anti-mouse antibody (Amersham, 1:2,000 dilution) to demonstrate the presence of a 66 kD osteopontin protein as has previously been reported [16]. The

![Epiluminescence photomicrographs of the same kidney specimens shown in Figure 1 using the antisense riboprobe for osteopontin.](image)

(A) An obstructed kidney at 96 hours after UUO exhibits the dramatic appearance of mRNA signal (which appears as green-colored grains) in proximal tubular epithelium (larger arrowheads) of the renal cortex and in the parietal epithelium of Bowman’s capsule (smaller arrowheads) of a glomerulus within the obstructed kidney cortex. The unobstructed kidney from the same animal (B) shows no presence of osteopontin mRNA signal (×640).

![Avidin-biotinylated immunoperoxidase labeling of renal cortex from the obstructed kidney at 24 hours post-left ureteral ligation using a monoclonal IgG mouse anti-rat osteopontin antibody (1:8,000 dilution).](image)

In A, there is an absence of staining within the glomerular tuft, however, the parietal epithelium of Bowman’s capsule stains intensely (small arrowheads). The larger arrowheads indicate staining within the proximal tubule emanating from this glomerulus. Other proximal tubules surrounding the glomerulus also label intensely for osteopontin. (B) Several proximal tubules are labeled intensely for osteopontin. The small arrowheads denote the proximal tubule brush border for orientation. As is evident, staining is throughout the entire cytoplasm, including the basolateral aspect of the proximal tubular epithelium (magnification ×640).
membrane was washed in an Amersham ECL detection mixture layered on the surface of the membrane. To measure differences between samples, the bands on the membrane were scanned for optical density and compared to other bands.

Cell culture and controlled cyclic cell stretch
Continuous cycles of stretch/relaxation utilizing a rat plasmid-transformed immortalized proximal tubule cell line (kindly provided by Dr. J. Ingelfinger, Harvard Medical School) [17] were performed. We selected this recently available rat cell line as it contains the renin-angiotensin components as well as expresses a variety of proximal tubule markers, including alkaline phosphatase, cytokeratin, carbonic anhydrase, and glucose transporter isof orm 2, while not expressing factor VIII [17]. In specific regards to the renin-angiotensin system, this cell line expresses protein and mRNA for angiotensinogen, ACE, the angiotensin II type 1 receptor, and renin [17]. These cells have been passaged over 45 times and retain the same morphological characteristics [17]. Thus, this cell line provides an excellent model system of rat proximal tubules, enabling the study of cell and molecular biology expression of osteopontin in response to mechanical cell stretch.

Rat proximal tubule cells were plated at a density of 3 to 5 \( \times 10^4/\text{ml} \) in DMEM containing 5% fetal bovine serum (FBS) using six-well plates with a flexible (experimental stretch/relaxation)-bottomed type I collagen-coated membrane (Flexcell Corp., McKeesport, PA, USA) according to our previously published methods [18]. The type I collagen substratum was selected since the rat cell line grew best to confluency with this matrix protein in contrast to laminin or fibronectin-coated plates. Cells were grown to confluency over 72 hours and then subjected to cyclic stretch (that is, stretch and relaxation/cycle) at 15 cycles/min for one hour with a one hour pre-treatment with losartan (10\(^{-5}\) M), using a computer-driven, vacuum-operated, stress-providing instrument (Flexcell Strain Unit FX-2000; Flexcell). An applied vacuum of 20 kPa was used resulting in an elongation of the membrane by approximately 15%. One six well plate constituted an \( N = 1 \). Studies were performed in triplicate. As a control, rat proximal tubule cells were grown to confluency on the similar type I collagen-coated flexible-bottomed plates, but were not subjected to repetitive cycles of stretch/relaxation although these cells were cultivated under identical conditions and durations to the cyclic/stretch counterparts.

RESULTS

In situ hybridization studies of osteopontin mRNA localization

Figure 1 is comprised of photographs of the autoradiograms of midcoronal kidney sections from a representative UUO animal at 96 hours after ureteral ligation, using the antisense rat osteopontin riboprobe with five days autoradiogram development. As is evident, mRNA signal is present only in the medulla of the CUK (Fig. 1A), which has been previously noted by Lopez et al [12] in normal rat kidney. In sharp contrast, the obstructed kidney from the same animal (Fig. 1B) exhibits diffuse, markedly up-regulated mRNA expression of osteopontin within the entire cortex as well as medulla. The sense riboprobe yielded only background signal while an RNase control (0.1 mg/ml; pre-incubation for 90 min) was entirely negative for mRNA signal.

Figure 2 is an epiluminescent photomicrograph of renal cortex from the same midcoronal sections obtained from a representative rat 96 hours after left ureteral ligation as shown in Figure 1 using the antisense riboprobe for rat osteopontin. Figure 2A, the obstructed kidney cortex exhibits the dramatic appearance of mRNA signal (which appears as green-colored grains) in proximal tubular epithelium adjacent to a glomerulus as well in the parietal epithelium of Bowman’s capsule of the glomeruli within the obstructed kidney cortex. In comparison the CUK (Fig. 2B) shows no presence of osteopontin mRNA signal.

Immunolocalization of osteopontin in rat renal cortex

Figure 3 demonstrates avidin-biotinylated immunoperoxidase labeling of renal cortex from the obstructed kidney of a rat 24 hours post-left ureteral ligation using a monoclonal anti-rat osteopontin antibody. In Figure 3A, there is an absence of staining within the glomerular tuft; however, the parietal epithelium of Bowman’s capsule stains intensely. As also shown in Figure 3, there is evident staining for osteopontin within the proximal tubules and labeling exhibits both apical and pan-cytoplasmic staining, including the basolateral aspect of the proximal tubular epithelium. As we have shown previously [2], the CUK and SOR renal cortex only exhibits weak osteopontin immunolabeling in the parietal epithelium of Bowman’s capsule.

Northern blot analysis of proximal tubular homogenates

In order to examine whether osteopontin mRNA expression is regulated, in part, through the angiotensin II type 1 receptor, we performed Northern blot analysis on normal rat proximal tubule homogenates stimulated with angiotensin II in the presence and absence of losartan. As shown in Figure 4, tubules incubated with angiotensin II (10\(^{-7}\) to 10\(^{-5}\) M) for one hour increased osteopontin mRNA expression by a factor of 1.3-fold, versus normal proximal tubules in medium only. In contrast, when tubules were pre-treated with losartan 10\(^{-5}\) M prior to the addition of exogenous angiotensin II (10\(^{-5}\) M), there was a normalization of osteopontin message. Also, since ACE inhibition may interfere with other hormonal systems (such as, kallikrein-kinin), the efficacy of losartan in normalizing osteopontin mRNA in angiotensin II-stimulated proximal tubules, reinforces the specificity of our postulate that the biological
activity of angiotensin II through its receptor (without altering kinins) up-regulates osteopontin expression. It should be noted that exposure of isolated proximal tubules to exogenous angiotensin II did not increase osteopontin mRNA expression in a dose dependant manner. An increased intensity of osteopontin mRNA expression may be observed with continued time and culture of the proximal tubular suspensions.

**Western blot analysis of proximal tubular homogenates**

Figure 5 is a Western blot of normal rat proximal tubules treated similarly as the Northern blot in Figure 4. As is evident in Figure 5, freshly proximal tubules incubated with angiotensin II (10^{-5} M) for one hour increased osteopontin protein expression by a factor of 1.9-fold in comparison to control tubules. This increased osteopontin protein expression was normalized following pre-treatment with losartan (10^{-5} M, lane 5) prior to addition of exogenous angiotensin II (10^{-5} M).

**Controlled cyclic cell stretch**

By Northern blot analysis, rat proximal tubule cells, which express all of the components of the renin-angiotensin cascade [17], exhibited a 2.1-fold increase in osteopontin mRNA levels following one hour of cyclic mechanical stretch, compared to control unstretched cells grown on the same substratum for identical durations (Fig. 6). Pre-treatment of proximal tubule cells with losartan (10^{-5} M) for one hour prior to cyclic mechanical cell stretch was found to normalize osteopontin mRNA expression (Fig. 6).

**DISCUSSION**

The present study underscores the importance of the renin-angiotensin system in regulating osteopontin expression in the renal cortex and in normal rat proximal tubules. The present study localized osteopontin mRNA and protein to the cortical tubules of the obstructed kidney. Stimulation of isolated proximal tubules with angiotensin II caused an increase in osteopontin mRNA and protein expression, as detected by Northern and Western blot analyses, respectively. Pre-treatment of proximal tubules with the angiotensin II type 1 receptor antagonist, losartan, prior to the addition of angiotensin II normalized osteopontin mRNA and protein expression. Furthermore, the early (that is, within 1 hr) increase in osteopontin mRNA expression in cultured rat proximal tubular epithelial cells in response to controlled cyclic stretch lends mechanistic insight into the pathobiology of renal cortical macrophage recruitment following ureteral ligation.

In a model of focal tubulointerstitial injury following direct intrarenal injection of angiotensin II [3], there is an increase in the focal expression of osteopontin mRNA and protein in renal cortical tubules and surrounding Bowman’s capsules, which precedes the macrophage influx. The macrophages localized almost exclusively to sites of tubular osteopontin, suggesting that inappropriate expression of osteopontin might be an important macrophage chemoattractant [3]. In fact, subcutaneous injection of osteopontin in mice stimulated a macrophage-rich inflammatory infiltrate [19]. In a recent study exploring the pathogenesis of chronic cyclosporine nephropathy, Pichler et al demonstrated that the degree of osteopontin expression correlated with the severity of interstitial fibrosis [4], and these authors concluded that the interstitial fibrosis in chronic cyclosporine nephropathy is associated with the osteopontin expression and macrophage accumulation. Our laboratory has recently noted up-regulated osteopontin mRNA and protein in temporal association with tubular cystic dilation and peritubular macrophage accumulation, preceding the development of interstitial fibrosis, in a hereditary rat model of autosomal dominant polycystic kidney disease [20]. Germane to the UUO model, we observed markedly up-regulated renal cortical osteopontin expression predominantly in proximal tubular epithelium in obstructed kidneys only, beginning as early as four hours...
post-unilateral ureteral ligation [2]. This demonstrates that the post-obstructed injury state elicits an extremely early chemoattractant response followed by a profound renal macrophage infiltrate. The mechanism for the increased osteopontin expression in the obstructed kidney only following ureteral ligation has been unclear; however, our data support a local intrarenal mechanism involving the renin-angiotensin II-generating ability of the proximal tubule.

Using a pig model, Froklaer et al demonstrated that the pig kidney responds to UUO by a net secretion of angiotensin II from the obstructed kidney and that a large part of the immunoreactive angiotensin II in the renal venous effluent originated from de novo intrarenal generation [21]. In a series of studies, Pimental et al [22, 23] and El-Dahr et al [24] demonstrated that acute UUO results in profound changes in the renal expression of the genes that encode for the components of the renin-angiotensin system. The net effect of these alterations is increased synthesis of angiotensin II in the renal venous effluent originated from de novo intrarenal generation [21]. The proximal convoluted tubule has the highest concentrations of angiotensin II receptors [29], with angiotensin II binding sites, predominately the type I angiotensin II receptor, present on the brush border and basolateral membranes of the proximal tubule [29].

Recent evidence suggests that angiotensin II, generated as a result of UUO, is a potent stimulator of interstitial fibrosis [8, 30]. Ishidoya et al demonstrated that angiotensin converting enzyme (ACE) inhibition at the beginning and throughout the course of UUO attenuated the increase in collagen IV mRNA, transforming growth factor-B (TGF-β), and interstitial matrix expansion [8]. A specific angiotensin II type 1 receptor antagonist also ameliorated extracellular
matrix expansion, but did not blunt macrophage infiltration [8]. These studies suggest that tubulointerstitial injury and the culmination of interstitial fibrosis in UUO appear to be dependent on the release of angiotensin II.

During the first hour of complete unilateral ureteral ligation, the increase in ureteral pressure following the onset of obstruction is instantaneously reflected in an increment in proximal tubular pressure, which produces a net decrease in the hydraulic pressure gradient across the glomerular capillaries [31]. Gottschalk and Mylle showed in diuretic rats that 10 to 15 minutes of UUO are sufficient to raise both ureteral and proximal tubular pressure to maximal values of 40 mm Hg [32]. However, as early as five hours after ureteral obstruction, proximal tubular pressure declines and glomerular filtration rate continues to fall as a result of reductions in renal blood flow and ultrafiltration coefficient [32]. It has also been postulated that following unilateral ureteral ligation, there is an increased cortical interstitial pressure [33]. We speculate that the transient hydrodynamic perturbations develop shortly after the onset of unilateral ureteral ligation in the obstructed kidney, which may produce a mechanical disturbance to proximal tubular epithelium. This mechanical disturbance might be membrane stretch, which increases the generation of components of the renin-angiotensin II axis within proximal tubular epithelium, resulting in autocrine stimulation of the type 1 angiotensin II receptor and consequent chemokine generation. This present investigation supports the above contention in that angiotensin II stimulation of normal proximal tubules resulted in increased osteopontin expression, which was abrogated by losartan. We have also recently shown that a similar process is involved in the expression of other proximal tubular chemokines, such as intercellular adhesion molecule (ICAM)-1 [14].

It can be argued that renal ischemia, due to reduced afferent arteriolar blood flow secondary to vasoconstriction, can be the stimulus for the up-regulated osteopontin expression. However, Kleinman et al have shown that following 40 minutes of left pedicle clamping with reperfusion at one and three hours post-ischemia, there were no differences in osteopontin expression between the ischemic kidneys at one and three hours of reflow and contralateral non-ischemic kidneys [34]. Three hours post-ischemia temporally correlates to when we have previously noted the beginning of up-regulated osteopontin expression in obstructed kidneys [7]. Furthermore, Kleinman et al noted that at 24 hours post-ischemia/reperfusion, osteopontin was markedly induced in medullary thick ascending limbs of the loop of Henle while there was only a modest amount of osteopontin observed in proximal convoluted tubules [34]. By 48 hours after ischemia/reperfusion, Kleinman et al noted that proximal convoluted tubule osteopontin expression normalized [34]. In the obstructed kidney, proximal tubular osteopontin mRNA and protein expression intensifies and continues to be up-regulated well beyond 96 hours after ureteral ligation. Thus, crucial temporal and spatial differences in osteopontin expression exist between the UUO and ischemia/reperfusion models, suggesting that factors other than ischemia (such as, mechanical stretch of proximal tubular epithelium) causes proximal tubular epithelial up-regulated osteopontin expression in our model.

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