Proximal Tubule Microvilli Remodeling and Albuminuria in the Ren2 Transgenic Rat

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Abstract

TG(mRen2)27 (Ren2) transgenic rats overexpress the mouse renin gene with subsequent elevated tissue Ang-II, hypertension, and nephropathy. The proximal tubule cell (PTC) is responsible for the reabsorption of 5-8 grams of glomerular filtered albumin (Alb) each day. Excess filtered albumin may contribute to PTC damage and tubulointerstitial disease. This investigation examined the role of angiotensin-II (Ang-II) induced oxidative stress in PTC structural remodeling; whether such changes could be modified with in vivo treatment with Ang type 1 receptor (AT1R) blockade (valsartan) or superoxide dismutase/catalase mimetic (tempol). Male Ren2 (6-7 week old) and age-matched Sprague-Dawley (SD) rats were treated with valsartan (Ren2-V; 30 mg/kg), tempol (Ren2-T; 1 mmol/L), or placebo for three weeks. Systolic blood pressure (SBP), albuminuria, n-acetyl-β-d-glucosaminidase (beta-NAG), kidney tissue malondialdehyde (MDA) were measured and PTC microvilli structure assessed using 60K transmission electron microscopy (TEM) images. There were significant differences in SBP, albuminuria, lipid peroxidation (MDA and Nitrotyrosine staining), and PTC structure in Ren2 versus SD rats (each p <0.05). Increased mean diameter of PTC microvilli in the Ren2-C (p <0.05) correlated strongly with albuminuria ($r^2 = 0.83$) and moderately with MDA ($r^2 = 0.49$), and there was an increase in the ratio of abnormal forms of microvilli in Ren2 rats when compared to SDC (p <0.05). AT1R blockade, but not tempol treatment, abrogated albuminuria and beta-NAG; both therapies corrected abnormalities in oxidative stress and PTC microvilli remodeling. These data indicate that PTC structural damage in the Ren2 rat is related to oxidative stress response to Ang-II and/or albuminuria.
Introduction

Chronic kidney disease (CKD) is increasing worldwide (15, 27). In the United States, it is estimated that 372,000 persons have end-stage renal disease (ESRD), and approximately 11% of adults are currently in the earlier stages of CKD (15). Principal causes of CKD leading to ESRD are type 2 diabetes mellitus (T2DM) and hypertension (HTN) (15, 27, 29, 31). Proteinuria is emerging as a major mediator of progressive renal disease leading to renal interstitial fibrosis and progressive renal injury (15, 29). The concept has been developed that excess protein filtered through the glomerulus exerts direct toxicity upon epithelial cells of the kidney proximal tubule (3, 5, 8, 28). Excessive filtered protein, particularly albumin (Alb), is thought to exert direct pro-inflammatory and pro-fibrotic effects on tubular epithelial cells (3, 5, 8, 13, 21, 29) and cause renal interstitial fibrosis (5, 8, 13).

Renal proximal tubular epithelial cells (PTC) are important for the reabsorption of most of the glomerular physiologically filtered Alb (8). Approximately 5 to 8 grams of Alb passes through the glomerular filtration barrier daily, yet only approximately 30 mg or less appears in the urine (8). Reabsorption of filtrate in the PTC occurs primarily by clathrin and receptor-mediated endocytosis (3, 5, 8, 13, 28, 35). Microvilli cover the apical portion of the PTC and provide the surface responsible for receptor-mediated endocytosis of Alb. Receptors that mediate this endocytosis include megalin and cubilin. Cytoskeletal remodeling involving actin and myosin mediated contraction facilitates the
formation of endocytic vesicles (3, 5, 8, 13, 28, 35). Alb is dissociated from these receptors and transported to lysosomes for degradation to amino acids, which are released through the basolateral membranes and absorbed into interstitial capillaries.

The mechanism by which exposure of excessive Alb leads to PTC injury, and the morphological and functional nature of this injury remains poorly understood. Compensatory hypertrophy of PTC in response to renal damage caused by HTN, hypoxia, and metabolic abnormalities appear to be an early process in the progression to tubular atrophy and tubulointerstitial fibrosis (18, 38). This hypertrophic process may be followed by apoptosis and other mechanisms leading to loss of PTC and/or microvilli (18, 20, 37, 38). There is accumulating evidence that the renal renin-angiotensin system (RAS) plays a pivotal roll in PTC maladaptive responses to various types of renal injury (4, 9, 16, 17). PTC produce and secrete angiotensin-II (Ang-II) into the lumen where its concentration (6-10 nmol/L) is at least 10 times higher than that in plasma (17). Ang-II (AT₁ and AT₂) receptors are located on both basolateral and luminal sides of the PTC (4, 9, 16). The critical location of Ang-II receptors and the high concentration of Ang-II suggest an autocrine/paracrine role for the RAS in the PTC. Further, in pathophysiologic conditions of albuminuria, as in T2DM or HTN, treatment with blockers of the AT₁R reduce the progression of renal disease (15, 27, 29, 43), as well as urine Alb loss (36). However, the precise mechanisms by which Ang-II mediates renal tubular damage remains poorly understood.

Excessive albuminuria has been reported to induce Ang-II production and increase Ang-II uptake by an AT₁R-mediated process (17). Increased renal Ang-II has been reported in systemically Ang-II infused rats and in the transgenic TG (mRen2)27
(Ren2) rat (19, 22, 41). The Ren2 rat overexpresses Ang-II in tissues and develops HTN, insulin resistance, and albuminuria (2, 22, 36). Investigations conducted in this and other hypertensive, insulin resistant, proteinuric rodent models has shown that Ang-II induced oxidative stress contributes to development of insulin resistance, HTN, albuminuria, and renal injury (2, 22, 36), and that these effects can be abrogated by AT1R blockade and anti-oxidant therapy (2, 26, 30, 36). Accordingly, in the present investigation we hypothesized that 1) the Ren2 rat would manifest oxidative stress mediated PTC injury in relation to albuminuria; 2) treatment of this model of Ang-II overexpression in vivo with either an AT1R blocker or a cell-permeable superoxide dismutase (SOD)/catalase mimetic (1, 32) would attenuate this Ang-II induced oxidative stress mediated PTC injury.

**Materials and Methods**

*Animals and treatments*

Male transgenic Ren2 rats and Sprague-Dawley controls (SDC) were obtained at 6 weeks of age and fed rat chow throughout the investigation. They were randomly assigned to treatment with valsartan (Ren2-V, SDV), tempol (Ren2-T, SDT) or placebo treated groups (Ren2-C, SDC). The treatment animals received valsartan (30 mg/kg/day) or tempol (1 mmol/L) in drinking water based on previous work (2), for 21 days prior to sacrifice. All protocols were approved by the University of Missouri and Harry S. Truman Veterans medical center animal care and use committee and housed/harvested in accordance with NIH guidelines.
Determination of Weight, Systolic Blood Pressure (SBP), Albuminuria, and N-Acetyl-β-D-Glucosaminidase (beta-NAG)

Restraint conditioning was initiated on the day of initial SBP measurement and then measured in triplicate, on separate occasions throughout the day, using the tail-cuff method (Harvard Systems, Student Oscillometric Recorder) on days 19 or 20 of treatment prior to sacrifice (2, 36) in addition to weights obtained.

A commercially available kit for rat urine Alb (Nephrat, Exocell Inc., Philadelphia, PA) was used to measure urine Alb. Urine was collected over a 24 hour period at the end of treatment and measured for levels of Alb normalized to creatinine. The Jaffé reaction on an automated chemistry analyzer (Olympus AU400, Olympus America, Dallas, TX) was used to measure urine creatinine (Cr). Results are reported as a ratio of urine Alb to Cr (mg/mg).

N-acetyl-β-d-glucosaminidase (beta-NAG) is a 140 kDa lysosomal enzyme present in high concentrations in PTC, but not typically excreted into the urine by normal intact PTC (25). Thus, increased beta-NAG in the urine is a potential marker for PTC injury (25). Urine excretion of beta-NAG was determined by colorimetric assay from Roche Diagnostics (Indianapolis, IN). Urine samples were first fractionated on a gel filtration column to remove NAG inhibitors and then assayed for NAG activity via a colorimetric assay on an automated chemistry analyzer (Olympus AU400, Olympus America, Dallas, TX). Beta-NAG excretion is expressed as mU of NAG activity.

Tissue Malondialdehyde (MDA):

To measure lipid peroxidation, tissue MDA levels were determined as a surrogate end-marker for oxidative stress (36). Butylated hydroxytoluene (BHT; 5 mM) was added
to kidney cortical tissue (100 mg) to prevent new lipid peroxidation. Samples were then homogenized in a buffer solution (0.25 M sucrose, 0.5 mM EDTA, 50 mM HEPES, protease inhibitors) on ice and centrifuged at 4°C at 15,000 rpm for 10 minutes. The supernatant was collected for MDA and O-phthaldialdehyde (OPA) protein assays. Supernatant (200 μL) was used to measure free MDA using a MDA-586 spectrophotometric assay kit (OxisResearch Biotech, Portland OR). Total protein was measured by OPA fluorometric assay and FLX-800 fluorometer (BioTek, Winooski, VT).

**Immunostaining of PTC with Nitrotyrosine:**

Nitrotyrosine staining was used to corroborate levels of lipid peroxidation in the PTC. Sections of kidney were deparaffinized, rehydrated, and epitopes retrieved in citrate buffer. Endogenous peroxidases were quenched with 3% H2O2 and non-specific binding cites were blocked with avidin, biotin, and finally with protein block (Dako, Carpinteria, CA). Sections were incubated with 1:200 primary antibody, rabbit polyclonal anti-Nitrotyrosine antibody (Chemicon, Cat# AB5411) then washed, incubated with the secondary antibodies, linked, and labeled (Strepavidin, Dako, LSAB+ Kit K0690) for 30 min each. After several rinses with distilled water, diaminobenzidine (DBA Dako, K3466) was applied for 10 min. Sections were again rinsed with distilled water, then stained with hematoxilyn for 1 min, rehydrated, and mounted with a permanent media. Slides were evaluated under a bright field (Nikon 50i) microscope and 40X images were captured with a cool snap cf camera. Images were analyzed and signal intensities measured with MetaVue (Boyce Scientific Inc. Gary Summit, MO).

**Transmission Electron Microscopy (TEM) Methods**
Renal cortical tissue from excised kidneys was thinly sliced and placed immediately in primary EM fixative (2% glutaraldehyde, 2% paraformaldehyde in 0.1 M Na cadodylate buffer, pH 7.35). A Pelco 3440 Laboratory Microwave Over was utilized for secondary fixation, with acetone dehydration and Epon-Spurr’s resin infiltration. Specimens were placed on a rocker overnight at room temperature, embedded the following morning, and polymerized at 60 degrees Centigrade for 24 hours. A Leica Ultracut UCT microtome with a 45 degree Diatome diamond knife was used to prepare 85 nm thin sections. The specimens were then stained with 5% uranyl acetate and Sato’s Triple lead stain. A JOEL 1200-EX transmission microscope was utilized to view all renal samples.

Three glomerular/proximal tubule fields were randomly chosen per rat in order to obtain three 60K images/kidney containing cross sectional views of the microvilli. PTC were visualized adjacent to glomeruli and were identified on the basis of their continuous microvilli at the apical surface. Then five (450 nm X 450 nm) images were cropped out of each 60K image obtained. These cropped images were then analyzed using Image J (a public domain Java image processing program made possible by the NIH). By viewing the microvilli in cross section in each cropped image, the diameter of the microvilli were measured and recorded in a blinded fashion (38). Additionally, abnormal forms as defined by a diameter greater than 100 nm were counted per each field (Figure 3). The value of 100 nm was chosen as a value approximately greater than 2 standard deviations above the upper limit of the range of 76.6 to 86.9 nm of normal proximal tubules observed in control animals.

RNA Isolation and Reverse Transcriptase/Real-time Polymerase Chain Reaction
RNA was isolated from renal tissue and measured for angiotensinogen, renin, angiotensin-converting enzyme (ACE), ACE2, neprilysin, AT$_1$R, and the mas receptor using the TRIZOL reagent (GIBCO Invitrogen, Carlsbad, CA), as previously reported (34).

**Statistical Analysis**

All values are expressed as mean ± standard error. Statistical analyses were performed in SPSS 13.0 (SPSS Inc., Chicago IL). Abnormal forms were evaluated via nonparametric binomial testing with SDC control values as the test parameter and a cut point of 100 as a value approximately greater than 2 standard deviations above the upper limit of the range of 76.6 to 86.9 nm of normal proximal tubules. All other variables were evaluated parametrically via ANOVA with Fisher’s LSD, as appropriate. Data from PTC measurements were then plotted against final systolic blood pressure, albuminuria, and MDA and then natural log (ln) transformed as appropriate for linear regression analysis. P <0.05 was considered statistically significant.

**Results**

**Systolic Blood Pressure (SBP), Albuminuria, and beta-NAG**

SBP were significantly higher in Ren2-C (n = 6) (192 ± 5.8 mmHg) compared to SDC (n = 4) (123 ± 5.5 mmHg) (p <0.05) (Table 1), and significantly lower with valsartan treatment (Ren2-V) (n = 6) (147 ± 6.1 mmHg) (p <0.05) but not improved with tempol treatment (Ren2-T) (n = 4) (188 ± 8.4 mmHg) (p >0.05). Consistent with prior observations that the Ren2 is insulin resistant, measured glucoses were higher in the Ren2 than SD (p <0.05). Similarly, albumin/creatinine ratio (Table 1) was higher in Ren2-C
(0.27 ± 0.02 mg/mg) versus SDC (0.06 ± 0.01 mg/mg) (p <0.05), and decreased with valsartan treatment (0.05 ± 0.02 mg/mg, p <0.05), but not tempol (0.31 ± 0.07 mg/mg) (p >0.05). Beta-NAG was not significantly elevated in the Ren2-C animal compared to SDC. However there were significant reductions seen with valsartan treatment (Table 1).

Oxidative stress:

A marker of lipid peroxidation, MDA levels were elevated in Ren2 (Ren2-C) kidney cortical tissue (0.56 ± 0.17 µg of MDA/mg of protein) compared to SDC (0.05 ± 0.01 µg of MDA/mg of protein) (p <0.05), and decreased in the Ren2-V (0.10 ± 0.01 µg of MDA/mg of protein) and Ren2-T (0.22 ± 0.12 ug MDA/mg protein) (p <0.05) (Table 1). Similar to MDA, there were significant increases in grey scale intensity measures of Nitrotyrsoine, another marker of lipid peroxidation, on PTC cross sections in the Ren2-C (53.2 ± 5.6 arbitrary units) compared to SDC (20.6 ± 3.6 arbitrary units) (p <0.05) that were decreased in both the Ren2-V (22.2 ± 1.8 arbitrary units) and Ren2-T (32.4 ± 5 arbitrary units) (each p <0.05) (Figure 1A and B).

Ultrastructure analysis:

The diameters of PTC microvilli were measured in cross section and compared between treatment groups (Figure 2 and 3). Ren2-C exhibited a significantly increased mean microvilli diameter (86.94 ± 0.25 nm) (Figure 3A) associated with albuminuria (r² = 0.83) and levels of MDA (r² = 0.49) (Figure 3B) when compared to SDC (76.58 ± 0.19 nm) (p <0.05). Treatment with valsartan (Ren2-V) and tempol (Ren2-T) resulted in a reduction of the mean diameter (75.85 ± 0.14 nm and 80.57 ± 0.22 nm; p <0.05, respectively). Even with the exclusion of abnormal forms from diameter analyses, the relationships among the groups remained significant (data not shown). Thus, the presence
of abnormal forms alone did not account for the diameter increase seen in Ren2-C animals. The number of abnormal forms as a ratio of abnormal forms/total number of microvilli was increased in the untreated Ren2-C rats compared to SDC (0.14 ± 0.01 vs. 0.03 ± 0.01) (p < 0.05) (Figure 4B). Treatment groups Ren2-V and Ren2-T demonstrated a significant reduction in the number of abnormal forms (0.03 ± 0.002 and 0.05 ± 0.01) (p < 0.05).

**Tissue mRNA for RAS:** There were expected increases in mRNA transcripts for AT1R (1.79 ± 0.20) and mas receptor (2.43 ± 0.04) in Ren2-C when compared to SDC (p < 0.05) and decreases with AT1R blockade (1.28 ± 0.18 and 1.38 ± 0.13, respectively; p < 0.05) and also tempol treatment (mas receptor, 1.87 ± 0.16; p < 0.05). Further, there were significant improvements with AT1R blockade in the Ren2-C mRNA transcripts of ACE2 (1.80 ± 0.20), renin (5.41 ± 1.10), angiotensinogen (1.43 ± 0.11), and neprilysin (1.61 ± 0.07) (each p < 0.05). Similar improvements were seen with tempol treatment in ACE2 (1.97 ± 0.18), neprilysin (1.81 ± 0.22), and angiotensinogen (1.57 ± 0.1) mRNA transcripts (each p < 0.05).

**Discussion**

This investigation demonstrated that the Ren2 rat, which manifests enhanced tissue RAS (2, 22, 36, 41), develops renal tubular structural abnormalities in conjunction with development of HTN and albuminuria. TEM analysis revealed that the proximal tubules of the Ren2 rat displayed substantial microvilli abnormalities when compared with age-matched SD littermates. These abnormalities were generally characterized by increased mean diameter and increased numbers of abnormal microvilli structures.
Further, the extent of the abnormalities of the Ren2 microvilli were directly related to quantitative increases in urinary albumin. These observations are in concert with prior reports that albuminuria may cause PTC injury (3, 5, 8, 13, 28).

Our current understanding of PTC damage in response to albuminuria or other insults (i.e. hypoxia and metabolic abnormalities) is very limited. However, the TEM abnormalities in the Ren2 rat are potentially consistent with loss of the cytoskeletal integrity necessary for maintenance of erect tubules (20, 37, 38). Indeed, it has been previously observed that actin and myosin microfilaments undergo depolymerization, fragmentation, and basolateral retraction from the apical region of the microvilli in conditions of ischemia and HTN injury (20, 37, 39). This loss of cytoskeletal integrity likely contributes to flaccid/folded microvilli. This alteration, in conjunction with apoptosis/necrosis, could account for the loss of microvilli and widening of remaining microvillar structures observed in this study. Finally, the observation that oxidative stress increased in the Ren2 as measured by MDA and confirmed with staining of nitrotyrosine in the PTC and that in vivo treatment with both valsartan and tempol abrogated the microvilli structural abnormalities may suggest a role for oxidative stress as a common converging process mediating PTC injury to both Ang-II and albumin excess in the Ren2 rat.

As previously observed in skeletal tissue (2) and cardiovascular tissue (34), there were increased oxidative changes (MDA) in the renal cortical tissue of the Ren2 rat. The HTN (22, 41), insulin resistance (2, 12, 34), and albuminuria (36) in this rodent (2, 12, 34) model are related to excess tissue Ang-II signaling through the AT_1R to increase oxidative stress. Our observed increases in lipid peroxidation, both in cortical tissue
MDA levels and more specifically on Nitrotyrosine staining of the PTC in the Ren2, suggests a role for oxidative stress in the PTC remodeling. That both AT1R blockade and tempol treatment decreased renal lipid oxidation, as well as PTC microvilli structural abnormalities, provides evidence for Ang-II/albumin induced oxidative stress as a convergent pathway in the pathogenesis of PTC injury in this rodent model of tissue RAS overexpression. Furthermore, it is also important to consider ACE2 and neprilysin activity in opposing the effects of Ang-II stimulation of the AT1R (7, 10, 36). We have previously observed that valsartan increased expression of ACE2 and neprilysin in the renal cortical tissue of Ren2 animals (36). We report here similar effects noted following *in vivo* treatment with tempol as well. Collectively, these observations suggest that diminishing oxidative stress improves relative ACE2 signaling in renal cortical tissue.

Ang-II generates superoxide anion (O\textsuperscript{2−}) and hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) in cardiovascular tissue (34) and the kidney (14, 26, 36). At normal physiologic concentrations, O\textsuperscript{2−} and H\textsubscript{2}O\textsubscript{2} can act as second messengers in Ang-II mediated signaling pathways, but in excess they mediate inflammation and cellular dysfunction (33). The tubular structural alterations were related to levels of MDA and albuminuria, and damage was ameliorated by either 3 weeks of tempol or valsartan treatment. Tempol is a SOD/catatase mimic, that attenuates development of HTN and vascular injury via scavenging of ROS, both O\textsuperscript{2−} and H\textsubscript{2}O\textsubscript{2} (1, 30, 32). Indeed, previous *in vivo* treatment with tempol has been shown to normalize vascular superoxide levels, blood pressure, and renal inflammatory responses in Ang-II infused hypertensive rats (1, 24). Although valsartan treatment abrogated HTN, albuminuria, and PTC injury; tempol exerted its protective effects on PTC despite not significantly lowering systolic blood pressure or
albuminuria. Ang-II has previously been shown to selectively increase $O_2^-$ and $H_2O_2$ in PTC via NADPH oxidase activation, independent of mitochondrial $O_2^-$ production (6, 11). In PTC, the flavoprotein inhibitor diphenylene iodinium, as well as, the antioxidant N-acetylcysteine, prevented membrane-bound NADPH oxidase activation and generation of ROS (6, 11). These data collectively suggest that an increase in ROS is the convergent pathway by which increased Ang-II and/or albuminuria causes PTC injury.

One limitation in the interpretation of these results is the lack of increase in beta-NAG in the Ren2 which has recently been reported as an indicator of tubular injury. However, results from other studies have been inconclusive on the contribution of beta-NAG to proximal tubule injury and the most promising papers have been in the area of diabetic nephropathy. Further, that beta-NAG was not increased in the Ren2 despite the structural changes may reflect its relative insensitivity in detecting early tubular injury. Another possible limitation is drawing a conclusion regarding the correlation between oxidative stress (as measured by MDA) and PTC injury with an $r^2$ of 0.49. Although not as strong of an association as seen with albuminuria ($r^2 = 0.85$), there is a modest correlation between level of MDA and PTC structural changes. Furthermore, our staining of the PTC with Nitrotyrosine, specific for oxidation, does support our conclusions. However, these associations are best corroborated with in vitro studies. Thus, we are currently investigating the impact of ROS generation on structural and functional alterations in proximal tubule cells in culture (23).

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**Figure Legends**

**Figure 1A and B:** A) demonstrates immunostaining with Nitrotyrosine as a marker of lipid peroxidation on cross sections of Proximal Tubule Cells (PTC). B) demonstrates grey-scale measures of intensity of the PTC images in A). *, p<0.05 Ren2-C vs. SDC; **, p<0.05 Ren2-C vs. Ren2-V and Ren2-T.
Figure 2A through D. Cross sections of proximal tubule cells (PTC) microvilli for animal from each treatment group: A) Sprague-Dawley Control (SDC) - Note the uniformity of the microvilli of the PTC. B) Ren2 Control (Ren2-C) - Numerous abnormal microvilli forms present. C) Ren2 Valsartan (Ren2-V) - Number and size of abnormal forms reduced. D) Ren2 Tempol (Ren2-T) - Abnormal forms are reduced, but to a lesser degree.

Figure 3A and B. A) Microvilli mean diameter is significantly increased in Ren2-C vs. SDC and is attenuated in the Ren2-V and to a lesser degree in the Ren2-T. *, p<0.05 Ren2-C vs. SDC; **, p<0.05 Ren2-C vs. Ren2-V and Ren2-T. B) Linear regression analysis of natural log (ln) transformed values for albuminuria (Alb/Cr ratio) and malondialdehyde (MDA), a surrogate marker for oxidative stress, versus microvilli diameter (MVD).

Figure 4A and B. Abnormal forms of microvilli in the Ren2 animal model. The abnormal forms in the male 11 week old Ren2 model are highlighted by black arrowheads. These elongated forms of micorvilli in the Ren2 Model are noted to contain actin cytoskeletal filaments at both poles (white arrows in the 120K image insert). These findings may indicate that the microvilli have folded over during the cellular remodeling of the actin cytoskeleton. B) Ratio of abnormal to normal forms is significantly increased in Ren2-C vs. SDC and is similarly attenuated by treatment with Ren2-V and Ren2-T. *, p<0.05 Ren2-C vs. SDC; **, p<0.05 Ren2-C vs. Ren2-V and Ren2-T.
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*, p<0.05 Ren2-C vs. SDC; **, p<0.05 Ren2-V, Ren2-T vs. Ren2-C; #, p<0.05 Control vs. Valsartan
A: SDC

Ren2-C

Ren2-V

Ren2-T

B:

Average Gray Scale Intensities

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A

B

y = 0.0727x + 4.4943
R² = 0.4868

y = 0.0874x + 4.5969
R² = 0.8338