MACULA Densa Cell Signaling

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Abstract Macula densa cells are renal sensor elements that detect changes in distal tubular fluid composition and transmit signals to the glomerular vascular elements. This tubuloglomerular feedback mechanism plays an important role in regulating glomerular filtration rate and blood flow. Macula densa cells detect changes in luminal sodium chloride concentration through a complex series of ion transport-related intracellular events. NaCl entry via a Na:K:2Cl cotransporter and Cl exit through a basolateral channel lead to cell depolarization and increases in cytosolic calcium. Na/H exchange (NHE2) results in cell alkalization, whereas intracellular [Na] is regulated by an apically located H(Na)-K ATPase and not by the traditional basolateral Na:K ATPase. Communication from macula densa cells to the glomerular vascular elements involves ATP release across the macula densa basolateral membrane through a maxi-anion channel. The adaptation of multi-photon microscopy is providing new insights into macula densa-glomerular signaling.

INTRODUCTION

Within the kidney, the juxtaglomerular apparatus (JGA) is the site for the formation and regulation of glomerular filtration. This hemodynamic-filtration unit is morphologically complex (Figure 1), consisting of a hemodynamic conduit that includes the terminal segment of the afferent arteriole, the glomerular capillary network, and efferent arteriole. Glomerular capillaries, which are surrounded by Bowman’s capsule, are also structurally and functionally connected with mesangial cells. In normal adult humans, filtration through this capillary network occurs at a rate of 180 liters a day, and this filtrate is modified and processed by passage through specialized tubular segments into final urine. A unique feature of each nephro-vascular unit is that near the terminal portion of the nephron, the tubule returns to and is closely associated with the JGA. At this site, the morphologically distinct tubular cells, called macula densa cells, face the JGA. These cells monitor tubular fluid flow and composition and send signals that regulate
renal hemodynamics and the rate of filtration; this process has been termed tubu- 
loglomerular feedback (TGF) (1–4).

Macula densa cells reside within the cortical thick ascending limb (cTAL), and it is this segment that is water impermeable and possesses the apically located, loop diuretic-sensitive, Na:K:2Cl cotransporter. The cTAL is responsible for the dilution of tubular fluid. Upon reaching the macula densa segment, reabsorption of NaCl results in a reduction of luminal sodium chloride ([NaCl]) concentration to as low as $\sim 25$ mM, as well as a reduction in luminal osmolality (5–6). The degree to which [NaCl] is reduced, however, is highly dependent upon flow rate, with elevations in flow rate resulting in increases in luminal [NaCl]. Thus perturbations that elevate glomerular filtration rate (GFR) lead to enhanced flow, an elevation in luminal [NaCl] at the macula densa resulting in the transmission of a TGF signal that causes vasoconstriction and return of GFR to normal (1–4, 7). Most studies indicate that TGF plays an essential role in the regulation of GFR and renal hemodynamics and therefore in the maintenance of salt and water balance (1). Over the past few years there has been intense interest in understanding the biology and functional characteristics of macula densa cells and their role in the cell-to-cell communication process that forms the TGF signaling pathway. In addition, other studies have focused on the involvement of macula densa cell signaling in the control of renin release, but this area is not reviewed here. Instead, we focus on what is currently known about macula densa cells and on the latest concepts regarding the TGF signaling process.

The TGF signaling pathway involves an initial change in luminal fluid composition that is detected by macula densa cells. Macula densa cells then signal the underlying mesangial cells, which leads to the propagation of signals to other JGA structures, most notably, to the smooth muscle cells of the afferent arteriole (1–4). Because under normal conditions, TGF-mediated changes in renal hemodynamics involves concomitant reductions in glomerular capillary pressure, blood flow and GFR, the effector site for TGF is generally considered to be the afferent arteriole; i.e., TGF vasoconstrictor responses do not occur at the efferent arteriole (8). Nonetheless, there may be involvement of intraglomerular elements that can modify capillary filtration, the consequence of which is reflected by TGF-induced changes in the glomerular capillary ultrafiltration coefficient (9–10).

Because initiation of TGF involves concomitant changes in luminal fluid [NaCl] and osmolality, work has focused on defining and characterizing the transport processes in macula densa cells and how these processes may be involved in the detection of changes in luminal fluid composition. In addition, early in vivo work (11) found that the loop diuretic furosemide blocked TGF responses, which clearly focused subsequent efforts in identification of how these cells transport NaCl and how transport-related events might lead to the activation of intracellular signaling/messenger systems (12).

Since extensive reviews in the Annual Review of Physiology in 1987 (3, 9), work in this area has greatly benefited by the continuing development of several techniques. Each nephro-vascular unit contains a macula densa plaque of
∼20 cells; these cells are inaccessible using standard in vivo techniques and thus microdissection and in vitro perfusion of the cTAL-glomerulus that contains the macula densa cells are necessary to obtain direct access. In combination with fluorescence microscopy, electrophysiology, and patch-clamp techniques, these approaches have provided substantial information concerning the transport characteristics of macula densa cells (10, 12–13). In addition, immunofluorescence work has been useful in providing evidence for the existence of specific transporter isoforms. Recently, knockout mice models have been used to test for the effects of specific deletions of transporters, etc., on TGF responses.

**Na:K:2Cl Cotransport and Membrane Potential**

Macula densa cells are exposed at the apical membrane to varying levels of luminal [NaCl] and osmolality based on the rate of delivery of fluid from prior nephron segments. At the apical membrane, there is a Na:K:2Cl cotransporter (NKCC2 or BSC1) that is the primary means for NaCl entry into macula densa cells (7). Currently, there are three splice variants of NKCC2 (A, B, and F) that vary in distribution along the TAL, as well as demonstrate differences in ionic affinities and regulation (14–17). Macula densa cells appear to predominantly express the B isoform of NKCC2 (14). In recent expression studies, this isoform has been shown to exhibit the highest affinity for Cl relative to the other two isoforms (16–17). This makes sense because the lowest values for luminal [NaCl] occur at the macula densa, which is located at or very near the end of the TAL. Thus the high-affinity–low-capacity of the B isoform of NKCC2 appears to be well suited for transporting NaCl in an environment where luminal [NaCl] fluctuates around a value of ∼25 mM. As estimated by Lapointe et al. (7), at this low level of luminal [NaCl], the macula densa cotransporter is still functioning to reabsorb NaCl; however, it is operating at or near equilibrium values. With increases in luminal [NaCl], the cotransporter exhibits functional saturation and maximal transport rates at approximately 60 mM [NaCl] (16–18). This finding is consistent with TGF studies in which maximal feedback-mediated changes in GFR and glomerular capillary pressure occurs when luminal [NaCl] is increased to ∼60 mM (19). The relationship between luminal [NaCl] and TGF responses is shown in Figure 2A.

Using the isolated perfused thick ascending limb with attached glomeruli, it was possible to impale macula densa cells with microelectrodes and measure the basolateral membrane potential (V_{bl}) to alternations in luminal [NaCl]. Both our laboratory (20) and Schlatter et al. (21) reported that V_{bl} was responsive to changes in luminal [NaCl]. We reported that there was a marked and sustained depolarization of V_{bl} of >30 mV, with increases in luminal [NaCl]. Because the cotransporter is electrically neutral, membrane potential depolarization most likely reflects intracellular accumulation of Cl, with Cl egress through a basolateral Cl channel. This model is supported by a number of findings including a predominant Cl conductance at the basolateral membrane (22), an estimate that intracellular [Cl]
Figure 2  The relationship between luminal fluid [NaCl] and (A) tubuloglomerular feedback responses using in vivo micropuncture (adapted from Reference 19). Feedback responses were assessed using stop flow pressure as an index of glomerular capillary pressure. (B) Basolateral membrane potential, measured with microelectrodes in the isolated perfused TAL-glomerular preparation (taken from Reference 20), and (C) macula densa cell [Na] measured with the fluorescent dye SBFI (taken from Reference 48).
is above equilibrium potential (23), and a finding that both furosemide and the Cl channel blocker NPPB hyperpolarize macula densa cells and block $V_{bd}$ changes with alterations in luminal [NaCl] (20, 21). It was also found that the degree of depolarization was dependent upon the level of luminal [NaCl] over the range of 10 to 60 mM. In fact, the relationship between $V_{bd}$ and TGF responses versus luminal [NaCl] were virtually equivalent, as shown in Figure 2B. The importance of macula densa membrane potential in TGF was further supported by the work of Ren et al. (24) in which TGF responses were assessed in vitro by measuring changes in afferent arteriolar diameter with alterations in luminal [NaCl]. With the use of the ionophores, nystatin and valinomycin, Ren et al. found that maneuvers assumed to depolarize macula densa cell membrane potential were associated with TGF signaling. Thus a simple model of apical cotransport of NaCl followed by exit of Cl via a basolateral channel and subsequent membrane depolarization serves as the foundation for further understanding the TGF signaling process. A current model for transport by macula densa cells is shown in Figure 3.

Apical K Conductance

As described for the TAL, Na:K:2Cl cotransport with its obligatory K transport site, requires a continuous supply of K for efficient transport activity. Because TAL luminal [K] is at or below plasma values (24–27), continued NaCl transport requires that K be continuously re-cycled from cell to lumen. K secretion from cell to lumen in TAL and in macula densa is thought to involve an inwardly rectifying

![Figure 3](image)

**Figure 3** Current model of channels and transporters that have been identified in macula densa cells. In addition, these cells appear to be permeable to water, which is distinctly different from the surrounding thick ascending limb cells.
K channel that has a conductance of \( \sim 20–50 \) pS. ROMK, a cloned K channel, has been identified immunologically in TAL and macula densa and may be responsible for all or part of apical K secretion (28–31). Previous patch-clamp studies using cell attached and inside-out patches by Hurst et al. (32) found a remarkably high abundance of K channel activity at the apical membrane of macula densa cells, at least relative to the TAL. This channel was inhibited by intracellular Ca and acidic pH but was insensitive to ATP. This latter finding is unusual because ROMK generally has been found to be inhibited by ATP. However, as discussed by Wang (31), ROMK may interact with associate proteins. Therefore, it is possible that this K secretory pathway may have a unique set of characteristics in relationship to macula densa cells. The reason for the high level of K channel activity in macula densa cells is not known; however, because of the unique manner in which macula densa cells transport Na and K (discussed in a subsequent section), a much higher level of K channel activity compared with that in the TAL may be necessary. In addition to K recycling, this K channel may be responsible for hyperpolarization and regulation of macula densa cells membrane potential. In particular, it may function in the cell hyperpolarization observed when luminal [NaCl] is reduced from high to low levels. Nevertheless, this K channel is essential in TGF responses. Previous in vivo micropuncture studies have found that pharmacological blockade of luminal K channels leads to attenuation of TGF responses (25, 27). Also, as recently reported (33–34), TGF responses and renal function are severely impaired in mice with Type II Bartters syndrome in which ROMK has been genetically deleted. Thus the addition of an apical K recycling mechanism also appears to be an essential component in the macula densa mediation of TGF signaling.

Na/H Exchangers

Along with the Na:K:2Cl cotransporter, previous work has established the existence of Na/H exchange activity in macula densa cells. Fowler et al. (35) were the first to report that increases in luminal [Na] from 20 to 150 mM alkalinized macula densa cells in excess of .17 pH units. These studies were performed using the isolated perfused TAL-glomerular preparation in combination with fluorescence microscopy. In this case, macula densa cells were loaded with the pH sensitive-fluorescent probe BCECF, and intracellular pH was monitored using either photometry or imaging analysis. Unlike cotransport, which exhibits saturation at \( \sim 60 \) mM luminal [NaCl], there was a linear relationship between luminal [NaCl] and macula densa cell pH up to 150 mM (35). Na/H exchange activity was verified by blocking apical Na-induced changes in cell pH with amiloride or derivatives of this compound. Currently, at least eight isoforms of the Na/H exchanger have been identified. NHE1 is the nearly ubiquitous housekeeping isoform, which functions in cell pH regulation, whereas the other isoforms appear to play varying roles in pH and cell volume regulation, as well as Na and bicarbonate transport (36). In many polarized epithelial cells, NHE isoforms are located at both the apical and the basolateral
membranes. In TAL, current evidence for the configuration of NHEs shows NHE2 and NHE3 located at the apical membrane and NHE1 and NHE4 located at the basolateral membrane (36–42). In the TAL, NHE3 functions in Na and bicarbonate reabsorption (41–43). Recently, work from our laboratory (44), using both functional and immunological techniques, has identified the presence of NHE2 at the apical membrane of macula densa cells but the NHE3 isoform was not found. The presence of NHE2 was firmly established based on the IC50s for both EIPA and HOE-694 and by using antibodies that were specific for the NHE2 isoform, as shown in Figure 4. Thus this isoform appears to be solely responsible for the luminal [Na]-induced alterations in macula densa cell pH.

At the basolateral membrane of macula densa cells, the presence of NHE4 was detected using antibodies directed toward this isoform. Interestingly, there appears to be virtually no NHE1 in macula densa cells as assessed using both inhibitors (this isoform is highly sensitive to amiloride and its derivatives) and antibodies (44). This finding is highly unusual, although the reason or the consequences of the lack of NHE1 in macula densa cells is presently unknown. As reported in the TAL, NHE4 is localized at the basolateral aspects of these cells but appears to be mostly associated with intracellular vesicles. In TAL, a stimulus such as hyper-osmolality or cell shrinkage is necessary to active NHE4 (44). In contrast, NHE4 is expressed in macula densa cells both in intracellular vesicles and at the basolateral membrane (see Figure 4). This expression of NHE4 at the basal-plasma membrane is further suggested by the findings that NHE4 activity appears to be constitutively active. Thus simply reducing the Na gradient across the basolateral membrane results in a profound acidification that can be blocked by very high concentrations of EIPA or HOE 694, which is consistent with the insensitivity of this NHE isoform to derivatives of amiloride. However, the activity of NHE4 can be greatly enhanced in macula densa cells by raising bath osmolality independently of [Na] so that it is stimulated by cell shrinkage, similar to what has been reported for TAL. The role of NHE4 in macula densa, or for that matter in other cell types, has not been resolved. Macula densa cells exhibit marked changes in cell shape and volume so it is possible that NHE4 may serve in the cell volume regulatory machinery. However, this notion needs to be examined experimentally.

The role of Na/H exchange in TGF signaling is not known. Work of Wang et al. (43), using inhibitors of NHE3 (S3226) and NHE2 (HOE 694), have recently demonstrated that bicarbonate reabsorption in TAL is unaffected by HOE 694, whereas transport is markedly inhibited by the NHE3 inhibitor. In addition, transgenic studies in NHE3-deficient mice have also shown that the absence of this isoform results in profound and deleterious effects on renal excretion of salt and water, GFR, and blood pressure (45, 46). There are enhanced TGF responses in this model, likely the result of NHE3-deficient-volume contraction, which has been shown to enhance TGF sensitivity. In contrast, NHE2 knockout mice do not exhibit observable changes in blood pressure or renal function, including Na excretion (46). Current evidence regarding TGF responses in NHE2 knockout mice
remains equivocal (J. Lorenz, personal communication). Thus the role of the NHEs
in macula densa function and TGF signaling remains to be determined.

Macula Densa Cell Sodium Regulation

As stated, cotransport of NaCl into macula densa cells leads to elevation in intra-
cellular [Cl] (47), which then exits across the basolateral membrane. The question
remains; what happens to cell Na? Cellular extrusion of Na occurs against its elec-
trochemical gradient, and the classic model by which this occurs is via basolateral
Na/K-ATPase. However, as reported a number of years ago, macula densa cells do
not have an abundance of this enzyme (9). In fact, early work indicated that the
level of Na/K-ATPase might be as little as 1/40th of that expressed in the TAL.
Thus there was some early indication that macula densa cells might regulate cell
Na in a novel manner. This idea was borne out in recent studies (48) that assessed
intracellular [Na] dynamics in macula densa cells using SBFI, a Na-sensitive flu-
orescent probe. Measurement of intracellular [Na] in macula densa cells in the
presence of 25 mM NaCl in the lumen and 150 mM NaCl in the bath averaged
around 30 mM. This was significantly higher than that found in the adjacent TAL
(∼13 mM) and is also higher than that reported in other epithelial cells (48). Ele-
vations in luminal [NaCl] to 150 mM resulted in large increases in intracellular
[Na] to around 70 mM, whereas much smaller changes were observed in the TAL.
One of the most interesting aspects of this study was the relationship between
macula densa intracellular [Na] and luminal [NaCl]. There was an almost linear
increase in intracellular [Na] over the range of 0 to 60 mM NaCl, with no further
increase in cell [Na] between 60 and 150 mM (see Figure 2C). The relationship
between luminal [NaCl] and cell [Na] was the same as that found for V_b and for
tGF responses. This finding is remarkable because other cells appear to precisely
regulate [Na] levels in response to external perturbations. The concept that has now
emerged is that intracellular [Na] (and most likely [Cl]) tracks or reflects luminal
[NaCl]. This makes sense in terms of the sensor function of macula densa cells;
i.e., to have intracellular [NaCl] reflect changes in luminal [NaCl] over the range
where tGF responses occur.

Using measurements of macula densa intracellular [Na], studies showed that,
in the presence of low luminal [Na], addition of furosemide lowered cell [Na]
and greatly inhibited (∼80 %) increases in [Na] with elevations in luminal [Na].
This residual increase in cell [Na] appears to occur via the Na/H exchanger, which
further substantiates the 80–20% split in terms of apical Na entry that had pre-
viously been suggested for the Na:K:2Cl cotransporter and Na/H exchanger (23,
48). Interestingly, the addition of basolateral ouabain failed to alter macula densa
[Na], whereas it was effective in raising TAL [Na] (48). Apical addition of ouabain,
however, was very effective in raising macula densa cell [Na]. These recent obser-
vations suggest that macula densa cells regulate cell [Na] via an apically located
colonic form of the H/K-ATPase that is sensitive to ouabain. As reported in expres-
sion studies, this member of the ATPase family not only transports H and K but
can also transport Na, albeit less efficiently than the Na/K-ATPase (49, 50). The
presence of this ATPase has been identified both functionally (48) and immunologically. Verlander et al. (51) have recently reported the presence of a HKα2c at the apical membrane of macula densa cells. Thus it is clear that macula densa cells possess an Na-H/K-ATPase at the apical membrane and that it can function as a Na efflux pathway. The presence and abundance of subunits of the Na/K-ATPase in macula densa cells is less clear. The α1-subunit of Na/K-ATPase appears to be virtually absent in rabbit macula densa cells, whereas it is detected at low levels in rat (44, 52). Recently, Wetzel & Sweadner (52) reported, in the rat, the presence of high levels of expression of the γ-subunit of the Na/K-ATPase in macula densa cells, but not in surrounding TAL. This subunit has been shown to reduce the affinity of this ATPase for both Na and K (52). What has emerged from this work is a new model for cell Na/K regulation. In macula densa cells, Na extrusion and K entry occur at the apical membrane via a Na-H/K-ATPase. The fact that intracellular [Na] tracks luminal [NaCl] may be due to the reduced efficiency of this pump for Na removal. The finding that low levels of Na/K-ATPase, along with the γ-subunit, are expressed at the basolateral membrane suggests that this ATPase does not greatly participate in Na dynamics under normal conditions. However, it is possible that physiological perturbations could increase the contribution of basolateral Na/K-ATPase to macula densa [Na] regulation. This notion may not be applicable to only macula densa cells, it may be more generalized with regard to cellular regulation of [Na]. Thus cellular regulation of [Na] may be controlled by the pattern of ATPase expression.

Macula Densa Cytosolic Calcium Regulation

As discussed above, there are multiple effects of increases in luminal [NaCl] on macula densa cells such as increases in cell pH, elevations in cellular [NaCl], and depolarization of the basolateral membrane potential. Recent work has also reported that elevations in luminal [NaCl] result in increases in cytosolic [Ca] (53). This report is somewhat controversial because a previous report indicated no luminal [NaCl]-dependent elevations in [Ca] (54). However, this discrepancy may be related to technical issues regarding prior exposure to conditions that may have caused sustained increases in [Ca] before the actual experiments were performed. Nevertheless, it is now clear that increasing luminal [NaCl] will, in fact, cause a modest elevation in macula densa [Ca], as measured using the Ca-sensitive fluorescence probe, fura 2. It would also appear that the increase in [Ca] is from depolarization-induced Ca entry through a calcium channel across the basolateral membrane. Increases in [Ca] can be blocked by high (1 µM) concentrations of the voltage-operated calcium channel blocker, nifedipine, whereas the calcium channel agonist BAY K 8644, added to the bath, directly increased macula densa [Ca]. Finally, increases in [Ca] were blocked either by the addition of furosemide to the lumen or by adding NPPB, the Cl channel blocker, to the bath (53). As reported by Lapointe et al. (55) in patch-clamp experiments, there is a ∼20 pS non-selective cation channel in the basolateral membrane of macula densa cells that is activated by depolarization and by elevations in [Ca]. This channel has a
moderate Ca permeability and is very similar to TRPM4, a member of the TRP family known to be expressed in kidney (55a). Although its physiological role is, at present, uncertain it may serve in helping to maintain $V_{bl}$ depolarization and may also participate in Ca entry. However, this channel is not nifedipine sensitive so it is not clear whether this is the primary Ca entry pathway that was suggested based on cytosolic [Ca] measurements. In other work, Liu et al. (56) reported the existence of ATP purinergic receptors, most likely of the P2Y2 variety, at the basolateral but not at the apical membrane of macula densa cells. Since recent work has shown the release of ATP across the basolateral membrane (see below), it is possible that during TGF signaling, ATP-activation of purinergic receptors helps to maintain elevations in [Ca]. Consistent with the sensor function of macula densa cells, sustained elevations in luminal [NaCl] results in sustained elevations in $V_{bl}$. The non-selective cation channel and the purinergic receptors may help in the maintenance of feedback signaling.

Regulation of Macula Densa Transport

Numerous studies have investigated the regulation of TGF under a variety of physiological conditions. In general, low-volume states such as dietary salt restriction lead to enhanced TGF responses. Among the many identified modulators of TGF responses, angiotensin II (ANG II) has been the most extensively studied (1, 57–61). ANG II AT1 receptors, which mediate the vasoconstrictive properties of this hormone, are located throughout the JGA, including arteriolar smooth muscle cells and mesangial cells, and are clearly expressed at both apical and basolateral membranes of macula densa cells (62). Previous in vivo micropuncture studies have shown that ANG II enhances TGF responses, whereas blockade or inhibition of the renin angiotensin system leads to a blunting of TGF (57). Schnermann et al. (58, 60) reported an almost complete blockade of TGF signaling in mice in which the renin angiotensin system had been disrupted either at the level of the AT1 receptor or by eliminating converting enzyme. Recent studies have focused on the direct effect of ANG II on macula densa cells. As shown by Wang et al. (63), luminal administration of ANG II enhances TGF responses in vitro as assessed by changes in afferent arteriolar diameter with increases in luminal [NaCl]. This effect was inhibited by co-administration of an AT1 receptor blocker. Other studies have examined the effects of ANG II on transport mechanisms of macula densa cells. Our laboratory (64, 65) found that ANG II, added at nanomolar concentrations to either the lumen or bath, stimulated both the apical and basolateral Na/H exchangers. This stimulatory effect was blocked by an AT1 receptor blocker, and furthermore, high micromolar concentrations did not stimulate or significantly inhibited exchanger activity. This inhibitory effect was not blocked by Candesartan, an AT1 receptor blocker, suggesting that high levels of ANG II might activate other receptors such as the AT2 receptor. However, Wang et al. (63) reported no effect of PD 01233190121B, an AT2 receptor blocker, on ANG II stimulated TGF responses. Other studies by Kovacs et al. (66) have also reported that ANG II stimulates the Na:K:2Cl cotransporter. Using measurements of cell [Na], it was also found
that low concentrations of ANG II stimulated cotransporter activity, whereas high concentrations failed to enhance it. Interestingly, in this study, addition of the AT$_2$ receptor blocker in the presence of micromolar concentrations of ANG II restored the stimulatory effects of ANG II.

Taken together, these findings suggest that ANG II may specifically enhance TGF responses by acting on macula densa cell transport. However, the cellular mechanisms by which ANG II affects macula densa cell function is not known. It has been suggested that ANG II receptors can be coupled to a number of different pathways, including adenylate cyclase, protein kinases A and C, phospholipase C and A$_2$, cytosolic Ca, and P-450 arachidonic acid metabolites. However, previous work found that cAMP inhibits in vivo TGF responses, and has a direct inhibitory effect on the macula densa Na:K:2Cl cotransporter (67). Therefore, the effects of ANG II to stimulate cotransport activity probably do not occur through the protein kinase A pathway. In addition, for reasons that are unclear, it has been difficult to obtain ANG II-induced Ca-transients in macula densa cells (P.D. Bell, unpublished observations). In terms of regulation of the Na:K:2Cl cotransporter, previous studies by Laamarti et al. (67) suggest that intracellular $[\text{Cl}]$ may play a key role in controlling or regulating cotransport activity. This is consistent with studies in other cells in which intracellular $[\text{Cl}]$ may lead directly or indirectly to phosphorylation of the cotransporter at least for the NKCC1, the basolateral form of the cotransporter (67). There are additional complexities, because macula densa cells express high levels of the neuronal form of nitric oxide synthase, which forms nitric oxide, and the COX2 enzyme, which helps metabolize arachidonic acid (68–72). It is beyond the scope of this review to delve into the details and regulation of these two systems, and the reader is referred to the review by Schnermann & Levine in this volume (10). However, it is likely that the inhibitor effects of nitric oxide on TGF may be due, at least in yeast, to inhibition of the cotransporter. It has also been demonstrated that nitric oxide inhibits Na:K:2Cl cotransport activity in macula densa cells (P.D. Bell, unpublished observations) and thick ascending limb (73), and it may inhibit other transporter and channels and interact with other intracellular messenger systems. In terms of COX2, current work is focused on its role in regulation of renin release. Evidence from putative cultured macula densa cells indicates that low ambient NaCl may increase prostaglandin production via a tyrosine kinase, ERK, p38 pathway (74). Preliminary work from our laboratory also supports the idea that macula densa cells can release prostaglandins (most likely PGE2) in response to a reduction in luminal [NaCl]. Additional studies are needed to clarify the roles of nNOS, COX2, and other pathways that play a significant role in modulating TGF signaling and responses.

MULTI-PHOTON IMAGING OF THE JUXTAGLOMERULAR APPARATUS

One of the exciting advances in macula densa-TGF signaling has been the recent application of two-photon excitation fluorescence imaging to the macula densa-JGA complex (75). This technique is a radical departure from traditional confocal
Figure 5 Two-photon photomicrograph of a living JGA preparation that is perfused both from the TAL and from the afferent arteriole (AA). This high-resolution section is taken at a depth of approximately 50 µM within the glomerulus (G) in a preparation that was loaded with the membrane staining dye, TMA-DPH.

microscopy and has the distinct advantages of markedly improved image quality and deep optical sectioning of living tissue. As recently reported (75), this technique allows for high-resolution optical sectioning of the entire glomerulus and macula densa-mesangial cell-afferent arteriole complex. Figure 5 is an example of such an optical section, showing the various structures of the JGA in a preparation in which both the TAL and afferent arteriole were perfused. This new technique has revealed several exciting and provocative findings, including the response of macula densa (MD) cells to alterations in luminal fluid [NaCl]. As shown most clearly in video images, there are increases in macula densa cell volume with concomitant increases in luminal [NaCl] and osmolality, conditions that mimic normal physiology. This increase presumably occurs as the result of NaCl entry and the movement of water into the cell. Other work, at constant luminal fluid [NaCl] but varying osmolality, has demonstrated water flow across the macula densa plaque and into the mesangial cell field. This work substantiates earlier studies that reported finite water permeability of the macula densa plaque (13, 76). Finally, TGF responses have also been assessed using the double-perfused TAL-glomerular preparation. It should be noted that prior in vitro (59, 61) studies have measured TGF responses...
as changes in afferent arteriolar diameter at a site that is close to but outside of the glomerulus. These studies have uniformly reported, at best, modest changes (~10%) in luminal diameter, with increases in luminal [NaCl]. With two-photon microscopy, it has been possible to obtain intra-glomerular optical sections of afferent arteriole immediately before it branches into the capillary network. TGF signaling with increases in luminal [NaCl] led to an almost complete closure of the intra-glomerular afferent arteriole. There appeared to be an almost sphincter-like activity at this site. This new finding suggests that the effector site for TGF signaling is primarily located at an arteriolar site that is adjacent to the macula densa cells and within the glomerulus. Thus two-photon microscopy offers exciting new possibilities to examine glomerular physiology, JGA function, and macula densa signaling.

MACULA DENSA CELL SIGNALING: ROLES OF ATP AND ADENOSINE

A lingering question that has remained in the field of TGF has been the nature of the signal that emanates from macula densa cells to initiate renal vasoconstriction and decreases in GFR. The current consensus is that a substance is released from the basolateral membrane of macula densa cells that then signals mesangial cells and/or afferent arteriolar smooth muscle cells. One reason for this notion is that there are no specialized connections, such as gap junctions, between macula densa cells and the underlying mesangial cells (77, 78). At present, there are two candidates (adenosine and ATP) for the mediator of TGF, although these two candidates are not mutually exclusive. There is evidence for an involvement of adenosine in TGF signaling (79–86). This comes from both pharmacological studies as well as from knockout mice studies. Brown et al. (80) and Sun et al. (81) have reported that mice deficient in the adenosine A1 receptor lack TGF responses. Ren et al. (83) and Thomson et al. (84) have reported that acute blockade of adenosine A1 receptors inhibits TGF responses. In addition, preventing variations in adenosine levels by addition of a constant level of adenosine administration in the presence of a 5'-nucleotidase inhibitor also greatly attenuates TGF efficiency. Although these results provide support for a role of adenosine in TGF, some uncertainties remain concerning whether it has a permissive or mediator role. First, mesangial cells (when studied in culture), which normally lie adjacent to macula densa cells, do not respond to adenosine with increases in cytosolic [Ca] (87). It is also clear that mesangial cells are activated by TGF and that, as shown in previous work (88), can act as a functional syncytium, thereby transmitting signals to the smooth muscle cells and intraglomerular elements. Second, the vasoconstrictive responses of afferent arterioles to adenosine are transitory and wane with continued administration of this nucleotide (85). Third, adenosine A2 receptors are also present, and activation of these receptors clearly results in vasodilation.

The other potential mediator is ATP (85, 89–93). Afferent arterioles express P2X receptors, which, when activated by ATP, result in sustained vasoconstriction.
As discussed by Navar et al. (1, 85) and Inscho et al. (90), there is also a body of work that has demonstrated the role of ATP and purinergic receptors in renal autoregulation of blood flow and GFR, which is likely a reflection, at least in part, of TGF. In addition, Nishiyama et al. (92) found that renal interstitial ATP levels correlate with autoregulatory responses. We have recently examined this issue (93) in patch-clamp experiments of the basolateral membrane of macula densa cells and have identified a maxi-anion channel at the basolateral membrane that is in the order of 300 pS. It is anion selective and, most interesting, in inside-out patches, conducts ATP. A similar channel, at least in terms of its biophysical properties, has been extensively characterized in C127 cells (94–95). In macula densa cells, this maxi-anion channel is dependent upon extracellular NaCl, and channel activity is greatly attenuated by removing NaCl. In other work, a biosensor probe was used to measure ATP release at the basolateral membrane of macula densa cells. This involved the use of a PC12 cell that has purinergic receptors (P2X) and, when activated by ATP, has channel properties and also allows for Ca influx. By placing a PC12 cell at the basolateral membrane, it has been shown that macula densa cells release ATP when there is a specific increase in luminal [NaCl]. No luminal [NaCl]-dependent release of ATP was seen from surrounding TAL cells. Thus macula densa cells release ATP at the basolateral membrane in a manner that is consistent with TGF signaling.

It is possible that ATP and adenosine are both involved in TGF signaling (85, 86). Obviously, once ATP is released, it can be broken down into adenosine. Therefore, it is possible that ATP is the initial signaling molecule and that adenosine, which

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**Figure 6** Current scheme for the transmission of tubuloglomerular feedback signals. 1. Luminal sodium chloride is transported into macula densa cells primarily through a Na:K:2Cl cotransporter; 2., 3. this results in increased cell [Cl], membrane depolarization and elevations in [Ca] and pH; 4. ATP release from macula densa cells via a maxi-anion channel; 5. ATP activation of P2 receptors in mesangial cells, and 6. Gap junction conductance to the smooth muscle cells of the afferent arteriole, ultimately resulting in vasoconstriction and decreases in glomerular filtration rate.
is subsequently formed, acts as part of the signaling cascade. However, it is very clear that mesangial cells do possess purinergic receptors and that ATP, but not adenosine, causes depolarization and increases in mesangial cell cytosolic [Ca]. As recently reported by Ren et al. (96), mesangial cells are critical for TGF signaling. These workers found that damaging mesangial cells with a Thy 1-1 antibody or disruption of gap junctions between mesangial cells and smooth muscle cells resulted in a loss of TGF responses. This supports the notion that transmission of feedback signals occurs from macula densa cells through the mesangial cell field and to the vascular smooth muscle cells of the afferent arteriole. In this scheme, release of ATP across the basolateral membrane of macula densa cells may be the critical step in transmission of feedback signals (Figure 6).

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Figure 1  (A) Depiction of a nephro-vascular unit which illustrates that the distal tubule returns to its own glomerulus and forms a close association. MD, macula densa cells; PT, proximal tubule; TAL, thick ascending limb; DT, distal tubule; CD, collecting duct. (B) Illustration of the relationship between the thick ascending limb with macula densa and the glomerular mesangial cells and vascular elements. This figure also illustrates the tubuloglomerular feedback signal pathway from macula densa to afferent arteriole. (C) Photomicrograph showing the isolated perfused thick ascending limb-glomerular preparation. The macula densa plaque is clearly visible.
Figure 4: Immunofluorescence of: (A) apically located NHE2, (B) basolateral NHE4, and (C) the lack of Na/K ATPase in macula densa cells (taken from 44, 48).