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Review

NGAL-Siderocalin in kidney disease ☆

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ABSTRACT

Kidney damage induces the expression of a myriad of proteins in the serum and in the urine. The function of these proteins in the sequence of damage and repair is now being studied in genetic models and by novel imaging techniques. One of the most intensely expressed proteins is lipocalin2, also called NGAL or Siderocalin. While this protein has been best studied by clinical scientists, only a few labs study its underlying metabolism and function in tissue damage. Structure–function studies, imaging studies and clinical studies have revealed that NGAL-Siderocalin is an endogenous antimicrobial with iron scavenging activity. This review discusses the "iron problem" of kidney damage, the tight linkage between kidney damage and NGAL-Siderocalin expression and the potential roles that NGAL-Siderocalin may serve in the defense of the urogenital system. This article is part of a Special Issue entitled: Cell Biology of Metals.

1. Iron and kidney disease

Changes in iron distribution have been documented in both acute kidney injury (AKI) and chronic kidney diseases (CKD). Kidney iron and urine iron increase in CKD due to glomerular dysfunction [1–3] which leaks transferrin and other serum proteins into the filtrate, which induce secondary tubular damage [4]. Iron appears in the urine from hemoglobin and myoglobin extravasation [5] and from damaged tubular cells as a result of chemotherapy (cisplatin [6]; doxorubicin [7]), ischemia–reperfusion injury [8,9] and transplant ischemia [10] as well as other forms of AKI [9,11–15]. In each case, "catalytic iron" is found in the urine and blood, and oxidized lipids are well documented. Preloading animals with iron [14] worsened these diseases and conversely chelating iron with desferroxamine (DFO), blunted the damage [6,16,13,17–19]. Iron catalyzed damage is thought to be one of the earliest events in kidney dysfunction and is likely to be important in other organs as well, including the heart [20] where the siderophore chelator, carboxymycobactin was said to be protective. "Ferriuria" may also stimulate urinary tract infections which could result in endotoxemia and profound ischemic damage. These data raise many questions concerning the sources, mechanisms of trafficking and recycling of iron in tissue damage. It is also possible that iron release is not regulated whatsoever.

In normal conditions, transferrin (Tf) regulates iron delivery in circulation by solubilizing ferric iron (which is otherwise entirely insoluble above pH4; Ksp:10^{-10} M–10^{-18} M) [21] and delivering iron to cells via the TfR1. Yet, whether Tf-TfR1 participates in all forms of iron transport in all extracellular spaces including the recovery of iron in tissue damage remains to be seen. In fact, non-transferrin mechanisms must be active under specific circumstances, as demonstrated by a number of examples, including the hypomorphic alleles of $Tf^{hpx/hpx}$ which are born iron overloaded [22–26], *Tfr1* knockouts [27] which can still initiate organogenesis, and *GFP-Tfr1*$^{-/-}$ + *Tfr1*$^{+/+}$ embryonic chimeras [28–30] which demonstrate *GFP-Tfr1*$^{-/-}$ cells maturing essentially into every component of the kidney, without the ability to capture iron from Tf. In sum, while the mechanisms of Tf-iron chelation and donation are established, there are specific instances which suggest non-transferrin molecules must play a role, but most of these remain to be discovered.

In this brief review, we have examined the biology of the gene lipocalin2, known in tissue damage studies as "NGAL" and for its capture of siderophores, as "Siderocalin (Scn)". NGAL-Scn protein has been intensely studied by clinical scientists, but few labs have evaluated its basic biology.

2. NGAL-Scn: baseline expression

Biological fluids contain very low levels of NGAL-Scn protein at steady state level. Serum contains approximately 20 ng/ml NGAL-Scn which is probably derived from neutrophils and from limited expression in liver, spleen and kidney [31]. Renal clearance is a major regulator of this steady state level, because circulating NGAL-Scn undergoes glomerular filtration due to its low molecular weight (23–25 kDa) and positive charge (pI > 7.4). Theoretically, the kidney processes 3.4–4 mg NGAL-Scn per day (20 ng/ml × 120–140 ml/min glomerular filtration rate, GFR) as a result of filtration [32] followed by capture by

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the proximal tubule, where it was degraded to a 14 kDa fragment in lysosomes. Endocytosis of NGAL-Scn from the apical (luminal) membrane is the most likely pathway of NGAL-Scn traffic because it appeared in the urine when the apical megalin receptor was deleted. Biacore based binding studies confirmed a direct interaction between NGAL-Scn and megalin fragments [33], consistent with capture of NGAL-Scn from the glomerular filtrate rather than from the basolateral (blood) side.

Similar to serum, urine contains approximately 20 ng/ml NGAL-Scn at steady state. The origin of this protein is not clear, but some of it may derive from serum NGAL-Scn ("sNGAL-Scn") that bypasses capture in the proximal tubule (approximately 1/200 molecules). Alternatively, NGAL-Scn might derive from neutrophils or even from bladder epithelia. However, given the data discussed below, it is most likely that urinary NGAL-Scn ("uNGAL-Scn") derives from a low level of expression in the native kidney at steady state. Indeed, weak *in situ* RNA reactivity can be seen in the collecting ducts of some kidneys.

3. Ngal-Scn in Kidney Disease

3.1. NGAL-Scn as the biomarker

NGAL-Scn came to the attention of clinical scientists when it was found to be markedly upregulated by tissue damage [34]. Hence, NGAL-Scn joins a large number of stress induced proteins, but only in a few cases have in-depth studies demonstrated a quantitative relationship between damage and the expression of the protein. The following "proof of concept studies" demonstrate a tight linkage between stimuli which initiate kidney damage and the expression of NGAL-Scn protein. Much of this work was completed in the urinary pool, but we suspect that similar data will emerge in ongoing evaluations of serum.

3.1.1. Does NGAL-Scn originate from injured nephrons or from bystander nephrons?

Whole organ studies, including reciprocal cross-transplants between NGAL knockout and wild type mice have demonstrated that NGAL-Scn derives from damaged kidneys. For example, when chimeric mice (consisting of wild type kidneys placed in NGAL knockout bodies) were treated with ischemia, uNGAL-Scn and sNGAL-Scn were expressed [35]. On the other hand, there was much less uNGAL-Scn when the NGAL knockout kidneys were placed in wild type mice and subjected to ischemia (the urinary level essentially remained in the normal range).

A similar conclusion comes from human transplantation studies: human uNGAL-Scn and sNGAL-Scn were proportional to the rate of recovery from delayed graft function after transplantation, meaning that the greater the dysfunction of the allograft, the greater the expression of NGAL-Scn [36,37], implying that NGAL-Scn derived from the damaged nephron. Likewise, a chimeric mouse consisting of TLR4 kidneys placed in null hosts resulted in the brisk expression of NGAL-Scn in response to LPS, but TLR4 deleted kidneys placed in wild type hosts failed to generate the protein (Paragas, unpublished). The most likely interpretation of both of these experiments is cell- or "nephron"-autonomous responses to damage *i.e.* that NGAL-Scn derived from the damaged nephron and in the case of LPS from kidney epithelia which expressed TLR4. Cell autonomy was conclusively shown in cells isolated from the medulla of the kidney: NGAL-Scn was expressed in response to typical stimuli which trigger renal disease *i.e.* depletion of ATP [38], exposure to H_2O_2 [39] or the exposure to bacteria [33]. These data confirm that autonomous responses of the nephrons generate the NGAL-Scn protein.

Microscopic analysis provided additional confirmation that damaged nephron generates NGAL-Scn. The induction of ischemia in one part of the kidney resulted in NGAL-Scn RNA expression in nephrons throughout that domain, but not in the adjacent non-ischemic domain. A specific temporal pattern was also seen: tubular expression extended in a cortical-medullary direction after the injury (Fig. 1). *In situ* hybridization using a paraffin technique localized NGAL-Scn RNA in tubules with morphological evidence of damage including intra-tubular casts. When a selected group of chronic kidney diseases were evaluated, the location of NGAL-Scn RNA also corresponded to sites of tubular disease. An animal model of HIV Associated Nephropathy [40] demonstrated NGAL-Scn expression in medullary microcysts in late stages of renal failure. In fact, NGAL-Scn was expressed at levels far in excess of any other gene that has been previously associated with hypoxia, ischemia and renal damage. Similarly, lining epithelia of Polycystic Kidney Disease [41,42] expressed NGAL-Scn at levels higher than most other kidney expressed markers of tissue damage. These data show that the damaged tubule generates uNGAL-Scn and sNGAL-Scn.

3.1.2. Does the transcriptional response of the kidney parallel NGAL-Scn in serum and urine, and is this response a reflection of the intensity of the inciting stimulus?

To investigate the quantitative relationships between the expression of NGAL-Scn in the kidney and the amount of NGAL-Scn protein in the urine, we created a bioluminescent mouse by placing Luciferase2-mCherry reporters in the lipocalin2 locus [33]. Luciferase2 expression, which we designated "kidney NGAL-Scn" was proportional to the dose of ischemia or the dose of LPS. In addition, kidney luminescence paralleled the amount of NGAL-Scn protein appearing in the urine in a dose-responsive fashion. Related findings come from human studies of unilateral *vs* bilateral urinary obstruction [43], as well as studies of patients entering emergency departments where acute kidney injury was quantified by the graded "R-I-F-L" [44,45] or "AKIN" stages [46]. In these cases, NGAL-Scn responded in a quantitative and dose dependent fashion (bilateral renal obstruction induced twice the level of unilateral disease; higher RIFL or AKIN designations induced progressively greater amounts of NGAL-Scn). Moreover the kinetics of NGAL-Luc2 and uNGAL-Scn protein were nearly identical in the onset of expression, the peak of expression and in the termination of expression. Taken together, these data show that dose of damage generates a graded NGAL-Scn response in kidney and, in turn, in urine.

Mori et al. suggested that the disruption of the megalin dependent endocytic system in the damaged kidney could theoretically result in a failure to capture sNGAL-Scn from the filtrate, and consequently enhance the appearance of uNGAL-Scn [47]. While still reflecting nephron damage, this result would differ mechanistically from data obtained with the NGAL-Luc2-mC mouse. The former reflects damage to a segment of the nephron (the proximal tubule) that does not normally synthesize NGAL-Scn, while the latter reflects distal tubular synthesis and secretion into the urinary tract. We suspect that proximal tubule bypass is most likely active in chronic kidney diseases, but perhaps is self limited in AKI where the GFR falls in the damaged nephron (by definition). In addition, coordination between NGAL-Luc2 and uNGAL-Scn and the data from the cross-transplantation experiments (above) argue in favor of intra-renal synthesis and secretion as a dominant, but perhaps not the sole mechanism of uNGAL-Scn accumulation (escape from the proximal tubule being the second mechanism).

3.1.3. Does the appearance of NGAL-Scn temporally correlate with the inciting stimulus and is its expression reversible?

Kidney Luc2-mCherry and uNGAL-Scn both demonstrated rapid and reversible responses to ischemia. Data in mice were similar to the responses of children undergoing surgery and neonates suffering clearly timed stimuli such as sepsis [42,48,49]; both Luc2-mCherry and NGAL-Scn protein appeared in blood and urine within 3 h after initiation of the stimulus and reversed when the stimulus was removed. The rapidity of the transcription and secretion into the urine differs from other urinary proteins but the specific cellular mechanisms that govern NGAL-Scn secretion have not been characterized.

The reversibility of NGAL-Scn expression can be used to follow the actions of medications which suppress AKI including HCO_3 and fenoldapam [50,51] in humans, CO donors and PARP inhibitors in mice [52], and antibiotics in cell models.

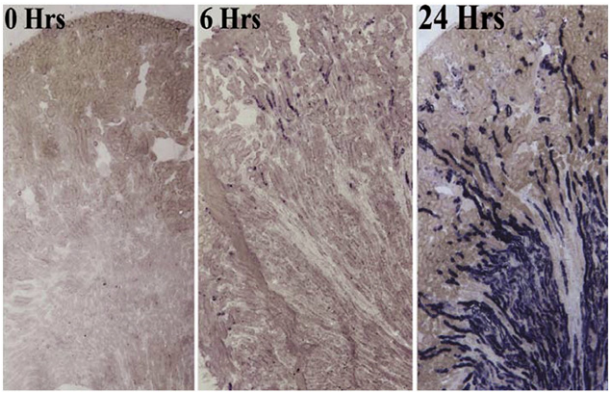

Fig. 1. *In situ* hybridization of NGAL-Scn in the mouse kidney after ischemia–reperfusion injury. Note the expression of NGAL-Scn in short cortical tubules by 6 h and throughout the TALH and collecting ducts at a later time point.

3.1.4. Does the NGAL-Scn predict clinical outcomes?

Many studies have shown that the presence of either serum or urine NGAL-Scn anticipates a severe course for the patient in hospital, including the need for dialysis and the possibility of death (NGAL-Scn at the time of presentation to the hospital presages 3–20 fold increased probability). In a metaanalysis, Haase, Bellomo showed that NGAL-Scn+ patients had more severe illnesses [53] than did the NGAL-Scn− patients and Hall et al. [54] showed that NGAL-Scn predicted poor outcomes. Ralib, and Endre et al. found that the daily amount of NGAL-Scn excretion correlated with the severity of outcomes [44] and NGAL-Scn at the time of discharge from the hospital presaged return to the hospital with clinically significant events [55]. In fact, patients who would otherwise have been overlooked in the emergency department because of apparently normal kidney function tests are at risk for rapidly progressive renal failure in the case that NGAL-Scn is expressed.

3.1.5. What is the role of NGAL-Scn in renal injuries?

As described in detail below, the canonical role of NGAL-Scn is to sequester bacteria derived iron-binding catecholate siderophores, particularly enterochelin (Ent). Crystallography and fluorescence quenching techniques demonstrated that NGAL-Scn binds *Ent* with high affinity extending over a broad range of biological pHs [72]. *In vivo*, the deletion of the NGAL-Scn gene led to increased mortality when the knockout mice were subjected to lab strains of bacteria [77]. Data relevant to the kidney is also now emerging. NGAL-Scn knockout mice cannot defend themselves against urogenital infections (Paragas et al., unpublished). Hence, NGAL-Scn is a directed response to infectious damage and consequently might also serve as a prophylactic in aseptic injury. On the other hand, NGAL-Scn may have a related function in aseptic injury such as scavenging iron with the metabolite catechol (discussed below and Summary and future experiments). It is also possible that NGAL-Scn plays a role which is not related to iron chelation per se. We initially isolated this protein by chromatography and a bioassay which demonstrated the growth and development of embryonic kidney cells [56]. Recent data from knockout mice also suggested a growth effect, because when damaged by chronic kidney disease, the knockout mice demonstrated reduced levels of epithelial proliferation [57]. Further analysis of these phenotypes may be achieved using experimental forms of NGAL-Scn which can traffic into the kidney tubule to inhibit bacterial growth, or to scavenge iron, or to induce epithelial growth.

3.1.6. Are characteristics of NGAL-Scn conserved across different populations and animal models?

In all models of human and mouse kidney diseases, the NGAL-Scn "monomer" or full length gene product is found in serum and in urine. In addition, the majority of the NGAL-Scn protein induced by acute renal failure in humans and in mice was a monomer. However, human NGAL-Scn contains an unpaired cysteine and can form not only monomers, but also dimers and hetero-multimers. These "high molecular forms" are found in chronic kidney diseases but not in acute kidney injury; their presence suggests either different cellular origins of the protein or variations in the metabolism of extracellular NGAL-Scn. For example, the dimer of human NGAL-Scn may arise from neutrophils [58] due to long storage of the protein in the environment of a neutrophil granule compared to the rapid secretion of NGAL-Scn monomer from epithelia. High molecular weight NGAL-Scn species also include cross linkage to transport proteins such as polymeric Ig receptors. These data are important because they suggest that the synthesis, trafficking and metabolism of "biomarkers" should be studied in clinical samples especially when the protein is known to be subject to proteolytic processing including Kim1 and Cyr61 and when the subject has chronic kidney disease.

Hence, while the expression and functions of NGAL-Scn are likely to be conserved across man and mouse, gene expression (the monomer) should be separately analyzed from protein metabolism (additional post-translational forms). These data are crucial in the analysis of chronic tissue injury because the specific form that is associated with epithelial injury (*i.e.* tubulointerstitial disease) must be the target of future epidemiological investigations [44].

4. Significance of NGAL-Scn expression data in clinical medicine

The intrinsic characteristics of NGAL-Scn listed above suggest that its measurement should be instituted in the diagnosis of tissue injury in human and in animal models [59,60]. In addition, the dose dependent and reversible characteristics of NGAL-Scn expression mean that it is useful to identify therapies that reverse the illness. In contrast, the rise in serum creatinine (sCr), a measure of functional deficiency rather than tissue damage, might be quite delayed in elderly patients with reduced muscle mass (creatinine derives from muscle). Also sCr levels may be elevated even in the absence of nephron damage as a result of normal functional changes, a condition which is classically called "prerenal AKI" [61–66]. Similar problems apply to the evaluation of chronic kidney disease. While uNGAL-Scn correlates with the severity of tubulointerstitial disease [44], which is the most important pathologic finding linked to CKD prognosis [67–70], sCr does not correlate well. Moreover there is a poor relationship between renal clearance [71] and sCr [72,73], and in 20% of patients with CKD [74] the relationship of $[sCr]^{-1}$ and disease progression does not hold at all [75]. Taken together these data illustrate that the diagnosis of renal tissue injury requires a single measurement of NGAL-Scn for its analysis, whereas multiple measurements of sCr separated in time are required to measure and characterize functional deficiencies.

5. NGAL-Scn binds co-factors which bind iron

5.1. Siderophores

A breakthrough in the understanding of the prominence of NGAL-Scn in kidney tissue injury was found in bacterially cloned NGAL-Scn by R. Strong and colleagues. NGAL-Scn is a member of the lipocalin superfamily which encodes a series of proteins that bind and transport small organic ligands within their internal calyx (Fig. 2). The lipocalin super family proteins bind a number of ligands: retinoids bind retinal binding protein, purpurin and rat epididymal RBP; pheromones bind major urinary binding proteins; colorants bind astaxanthin, and heme binds nitrophorins and α1-microglobulin. Goetz et al. [76,77] cloned NGAL-Scn in Gram− bacteria and identified a bacterial compound called enterochelin (Ent) bound within the protein's calyx. Ent is an organic molecule that bacteria utilize to capture iron from the extracellular media. It is composed of three 2,3 dihydroxybenzoates (catecholcarboxylic acids) linked by a tri-serine lactone backbone [78] so that its 6 hydroxyl groups form a "hexadentate" co-ordinate of ferric iron. R. Strong identified 3 positively charged amino acids in the base of the

calyx which provided 3 sites of cation–π and Coulombic interactions with Ent's catecholate rings, positioning their 6 hydroxyl groups to interact with iron. The amino acids are critical because their mutation diminished Ent:Fe binding. Complexes of NGAL-Scn with Ent:Fe (NGAL-Scn:Ent:Fe) could form in the bloodstream [79] followed by their rapid clearance by macrophages in liver and spleen and by filtration and tubular destruction in the kidney. Fe delivery to the proximal tubule by NGAL-Scn:Ent:Fe could be visualized using ^{55}Fe and radioautography. The pathway depended on megalin, since modification of megalin recognition resulted in the appearance of Fe in urine [75]. Hence, the binding of Ent to NGAL-Scn not only sequestered the siderophore but resulted in its clearance in the kidney. Because sNGAL-Scn levels can rise 100 fold to >2000 ng/ml, as much as 300–400 mg NGAL-Scn/24 h, containing nearly 1 mg of iron can theoretically traffic to the kidney for clearance and recycling, provided that Ent is fully saturated and glomerular filtration is maintained.

The interaction of Ent with NGAL-Scn directly blocked the capture of iron by bacteria: NGAL-Scn delayed bacterial growth, but an oversupply of iron or the expression of siderophores [80] which do not recognize NGAL-Scn, rescued bacterial growth. Consistently, the deletion of NGAL-Scn by homologous recombination accelerated the growth of Ent dependent bacteria, resulting in sepsis and heightened mortality [81,82]. Together, these data describe an interaction of mammalian NGAL-Scn protein with a bacterial product (Ent), and the subsequent clearance of NGAL-Scn:Ent:Fe by the proximal tubule.

It is likely that observations from systemic sepsis will also apply to the abundant NGAL-Scn expression within the urinary system, with a number of important caveats. uNGAL-Scn was significantly elevated by community acquired Gram$^-$ urinary infections according to a post-hoc analysis of patients in emergency departments [42] and other published cohorts [83,84]. Uropathogenic *E. coli* were the overwhelming culprits (70–95% of the cases) and most of these bacteria relied on catecholate-siderophores to capture iron [85,86] suggesting that NGAL-Scn should play a critical role in urinary defense. However, NGAL-Scn has been best characterized with *E. coli* sHB101 and H9049 which depend solely on *Ent*, whereas urinary bacteria potentially have multiple mechanisms of iron capture such as Salmochelin (*Iro*), *Salmonella* iron transport (*Sit*), Aerobactin (*iutA*), and Hemin uptake (*Chu*), which are NGAL-Scn resistant, meaning that direct tests of urinary inhibition are required. In addition, the sources of uNGAL-Scn could be more complex than kidney ischemia, because both bladder and kidney epithelia may be involved in the response to urinary bacteria [87]. There are also many apparent differences between known urinary antimicrobial peptides and NGAL-Scn which require further analysis. For example, unlike the well known antimicrobial proteins [88–90] the cathelicidins (hCAMP, mCRAMP), the defensins (HBD1 [91,92]), Tamm–Horsfall [93–97] and Lactoferrin [92], which are expressed constitutively, uNGAL-Scn is not likely to play a large role at steady state because it is expressed only at very low levels (20 ng/ml). In addition, while many of these proteins are modulated only to a small degree by infections (cathelicidin by 3–8 fold; THP and lactoferrin are not stimulated by infections [84,98–101] and the AMPs and lactoferrin remain in the low ng/ml range even after stimulation), NGAL-Scn was intensively upregulated by significant infections (Fig. 3) [102–106] as well as aseptic stimuli. Consequently the mechanisms of expression and induction apparently differ between NGAL-Scn and other antimicrobial peptides even though NGAL-Scn originates from the same cells as many of these proteins. Also additional analysis of the molecular characteristics of the NGAL-Scn:Ent interaction is required to determine whether Ent can be chelated in the harsh environment of the distal urinary tract (pH4.5; osmolarity 1200 mOsm/l; high concentrations of urea). It is known that similar to other urinary defenses (Defensin HB1 is active at pH5.5; lactoferrin is active at pH 3.0) NGAL-Scn:Ent:Fe binding is unusually pH insensitive.

In sum, human and mouse studies have shown that NGAL-Scn is intensively upregulated by significant infections that impact the kidney (200–20,000 ng/ml, Fig. 3). For example, patients entering emergency rooms demonstrated a linkage between spontaneous, community-acquired sepsis and uNGAL-Scn expression [62]. In neonates suspected of sepsis only those neonates who were truly infected (*i.e.* blood cultures later identified a pathogen) expressed high levels of uNGAL-Scn, but those neonates who were not infected (*i.e.* blood cultures were sterile or pathogen free) did not express uNGAL-Scn [77,102,107]. Consistently, cecal ligation and puncture models produced a 531 fold increase in kidney NGAL-Scn (Paragas and Barasch unpublished). Together, these data imply that the release of NGAL-Scn into the urinary system is a major response of the kidney to systemic infection and potentially local urogenital infection as well.

5.2. Extended function: catechol:iron?

Structural analyses by Strong and co-workers supported the idea that NGAL-Scn might bind additional ligands. First, Ent failed to fill the NGAL-Scn calyx, indicating that additional ligands are possible [73]. Second, NGAL-Scn had at least one additional ligand, a siderophore from mycobacteria called carboxymycobactin, which is structurally dissimilar to Ent. Third, a related member of the lipocalin superfamily, lipocalin1, can accommodate a variety of siderophores [108]. Fourth, while Ent is not synthesized by mammalian cells, it is a composite of well known functional groups such as hydroxybenzoates and hydroxybenzenes which are found in mammalian serum and urine. These data infer that NGAL-Scn

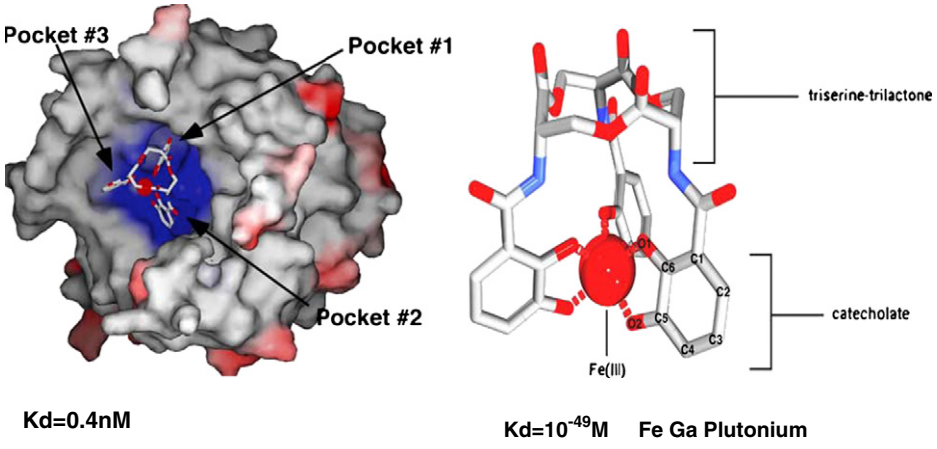

Fig. 2. Bacterial Enterochelin is composed of a triserine lactone backbone which coordinates three catecholate groups to form a hexadentate site that ligates a single iron atom. Iron–enterochelin complex binds the calyx of the lipocalin. The active site of NGAL-Scn is composed of three positively charged amino acids (R81, K125 and K134, where R is arginine and K is lysine). The binding is pH insensitive.
Molecular models courtesy of R. K. Strong, Fred Hutchinson Cancer Research Center, Seattle.

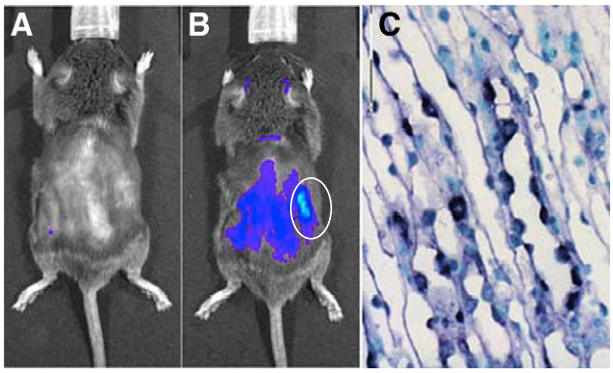

Fig. 3. NGAL-Scn-Luciferase2 (B) and NGAL-Scn RNA (C) expression detected after a dose of Lipid A. Note the kidney (white circle) and diffuse expression of NGAL-Scn-Luc2 (B) after the introduction of LipidA. (A) The same mouse before treatment. (C) Intercalated cells of the collecting ducts (alternating blue cells) respond to Lipid A and express NGAL-Scn RNA.

may bind additional ligands, other than Ent and carboxymycobactin, and that these ligands may derive from a variety of sources. Nonetheless, to date, few candidates have been proposed.

Given the extensive expression of NGAL-Scn in rodent and human urine subjected to either septic or aseptic renal damage, we used urine as a source of ligands. First, a paper chromatography assay demonstrated that while $^{55}Fe^{3+}$ precipitated in water, the addition of low molecular weight filtered urine or candidate urinary compounds solubilized $^{55}Fe^{3+}$. Also, their addition to a mixture of NGAL-Scn and $^{55}Fe^{3+}$ resulted in stable complexes similar to the addition of Ent. The urinary compounds that contained the dihydroxybenzene (catechol) functional group demonstrated saturation of NGAL-Scn (as measured by iron retention), and competition with a 50 fold excess of Ent:Fe^{3+} suggested that these compounds potentially occupy the same sites in the NGAL-Scn calyx as Ent. Simple catechol (1,2-dihydroxybenzene), pyrogallol (1,2,3-trihydroxybenzene), and 3- or 4-methyl catechol were the most active compounds and were stable during 20 h of H-NMR inspection. Using fluorescence quenching and other spectroscopic techniques [72] and structural studies, Strong and Raymond found that the catechols could add to the calyx in a stepwise fashion (*bis*-catechol:Fe^{3+} first, followed by the recruitment of a third catechol to form *tris*-catechol:Fe^{3+}) resulting in hexadentate coordination of iron. Most interestingly, catechol had a poor affinity for NGAL-Scn but in the presence of iron, the complex formed in the calyx at much higher affinity (2.1 nM and 0.4 nM). The catechols located at the same site where Ent bound (between the side-chains of residues K125 and K134), explaining their competition [109]. These data contrast with two other publications suggesting that norepinephrine (a 3,4 dihydroxycatechol) [110] and the polyphenol 2,5 dihydroxybenzoic acid can serve as NGAL-Scn ligands for iron [111]. Norepinephrine might have some NGAL-Scn iron binding activity (50 nM) but it is very reduced compared to 2,3 dihydroxy-catechols, and 2,5 dihydroxybenzoic acid lacks the iron-coordinated ortho-diol needed for strong chelation at neutral pH.

NGAL-Scn:catechol:Fe complex could form in mice from its component parts and quench the reactivity of catechol mediated Fe^{3+} reduction, and H_2O_2 mediated oxidation. The complex was stable for travel to the kidney and liver and bone marrow. This was particularly evident in the kidney, where the complex could be visualized at the apical membrane of the proximal tubule by radioautography. Its iron was likely to be recovered during the process of endocytosis in acidic endosomes of the proximal cell, because unlike NGAL-Scn:Ent, some catechols are pH sensitive (catechol > pyrogallol) [112]. This can be modeled in kidney cell lines expressing megalin where iron capture can be measured. In contrast to these data, transferrin-^{55}Fe demonstrated much less targeting of the kidney.

Catechol was first identified by von Euler. While difficult to measure because of oxidation upon storage, catechol (up to 150 μM) [113–117] and 4-methylcatechol (30 μM), and pyrogallol (500 μM) [109,118] are known to be abundant in the urogenital system. The majority of the catechols are sulfated and deactivated and this may take place in the kidney before excretion [119] (free catechols constitute 3–5% of total or 1–5 μM) [120]. However, the exact origin and metabolism of these molecules require further testing, but dietary plant quinic, shikimic [121] and 3,4-dihydroxybenzoic acids [117,122] and related hydroxybenzenes [109,114] or tyrosine metabolism to phenol may be their sources. It appears that enteric microorganisms participate at one or more steps of these pathways because sterilization of the gut reduced catechol excretion [123].

6. Summary and future experiments

NGAL-Scn is a rapid response gene whose expression is driven in a dose dependent manner by stimuli which generally induce tissue damage [42,62,75,124]. Its abundance at both the RNA and at the protein level have intrigued clinical scientists who have confirmed that its expression and its abundance reflects toxic stimulation just hours before gene expression. The rapidity and the intensity of its expression have been useful for identifying patients at particular risk for the onset or progression of kidney dysfunction, and for serious medical complications (dialysis, death). These basic clinical observations have been reproduced in many patient populations and animal models. Given that many stimuli activate NGAL-Scn, the most important next experiment is to determine the mechanism of signaling from clinically relevant stimuli to cells of the distal kidney. These experiments may come from further patient observations of nephrotoxins which target specific domains, or from segment specific methods of nephron damage as well as studies of the NGAL-Scn promoter.

There are also many unknowns concerning the trafficking of NGAL-Scn. A serum pool terminates at the proximal tubule megalin receptor, but an additional receptor 24p3R has been proposed to participate in NGAL-Scn capture as well [125,126]. It will be exciting to see if its expression is important for recycling of NGAL-Scn ligands at the level of the proximal tubule or whether 24p3R is a highly unusual component of a protein recovery system in the distal nephron. Our data also suggest that the urinary pool derives mostly from tubular cells of TALH and collecting ducts but protein secretion from the nephron is not well studied. How NGAL-Scn traffics apically or basolaterally or both is relevant for our understanding of the clinical data as well as to identify cellular targeting mechanisms in nephron cells. These mechanisms are of particular interest in the intercalated cell which undergo reversals of protein polarity to achieve acid–base homeostasis [127].

Most importantly, there has not been an adequate explanation for the abundant expression of serum NGAL-Scn in different kidney diseases. Among the many possible explanations (see below), bacteriostasis has the greatest support from structure–function data and from NGAL-Scn knockout data. However what happens to the siderophore complex upon clearance in the proximal tubule remains to be seen. One paper indicates that Ent itself induces cytokine expression [128], but it is not known whether trafficking of NGAL-Scn:Ent to the proximal tubule has this property; this type of signaling would be a novel mechanism of inducing a renal response to systemic infection.

Bacteriostasis is also the most likely role of urinary NGAL-Scn, but it remains to be seen if typical urinary pathogens are sensitive to NGAL-Scn. Also, while systemic sepsis can activate NGAL-Scn expression through TLR expression, the mechanism of NGAL-Scn induction in the urogenital system is not fully known, and may involve novel TLR11 [129] or other receptors.

Given that kidney NGAL-Scn is abundantly expressed in aseptic injury, it has also remained unclear whether NGAL-Scn plays a "prophylactic" role limiting the eventuality that the damaged kidney might be colonized by ascending bacteria or whether NGAL-Scn has additional

activities, such chelation of catechol or its family members when iron is released from damaged cells. Catechol can mobilize Fe^{3+} from other proteins [130] and the catechol:Fe complex may then participate in iron recycling that stimulates the Fenton reaction [131,132]. In contrast, ligation of catechol:iron within NGAL-Scn calyx may permit transport and excretion of catechol:iron in a less reactive form. Consequently, targeting of the kidney by both catechol and NGAL-Scn might clear catechol and iron, as well as Ent and iron [75], removing potential iron donors for microbes both at the level of filtration and in the distal nephron. This idea is in principle similar to the proposal of Jones and Cerami et al. [133] who found that low molecular weight iron carriers in mammalian organs can donate iron to microbes. Further testing of these speculative hypotheses should include isolation of NGAL-Scn complexes from urine or serum to examine occupancy of the calyx, measurement of redox damage in NGAL-Scn knockout mice and oral antibiotic treatment and dietary restrictions to decrease catechol metabolites. It should also be possible to create forms of NGAL-Scn that retarget iron from the proximal tubule to the urine as a test of the role of NGAL-Scn as an iron scavenger in the damaged nephron.

Finally, it should be pointed out that many groups have shown that NGAL-Scn is a growth regulator. We initially utilized the development of rat kidney progenitors to purify growth factors, identifying NGAL-Scn. A number of other groups have also ascribed epithelial growth to NGAL-Scn [134] including in kidney epithelia [135]. In contrast, there are reports that the withdrawal of growth factors from hematopoeitic cells resulted in NGAL-Scn expression followed by apoptosis [136]. A similar correlation was documented in the involuting breast and the post-partum uterus [137–139] where NGAL-Scn was expressed during the reabsorption phase. Likewise, some authors have found that NGAL-Scn is stimulated by pro-apoptotic factors [140] and that it directly induces apoptosis [132,141] in myeloid and early erythroid progenitors and in the kidney glomerulus [142], while other authors believe that NGAL-Scn, expressed during apoptosis, has a survival activity, because knockdown of NGAL-Scn expression enhanced apoptosis [136]. Taken together these data suggest that NGAL-Scn has growth activity that is active in the kidney, but its mechanism remains unclear. Resolution of these findings must await confirmation of the proposed receptor 24p3R or identification of additional novel receptors and their signaling mechanisms as clues to growth effects. However, at this time, siderophore chelation and potentially iron scavenging and trafficking remain the most investigated activities of NGAL-Scn.

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