HNF1β Is Essential for Nephron Segmentation during Nephrogenesis

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ABSTRACT

Nephrons comprise a blood filter and an epithelial tubule that is subdivided into proximal and distal segments, but what directs this patterning during kidney organogenesis is not well understood. Using zebrafish, we found that the HNF1β paralogues hnf1ba and hnf1bb, which encode homeodomain transcription factors, are essential for normal segmentation of nephrons. Embryos deficient in hnf1ba and hnf1bb did not express proximal and distal segment markers, yet still developed an epithelial tubule. Initiating hnf1ba/b expression required Pax2a and Pax8, but hnf1ba/b-deficient embryos did not exhibit the expected downregulation of pax2a and pax8 at later stages of development, suggesting complex regulatory loops involving these molecules. Embryos deficient in hnf1ba/b also did not express the irx3b transcription factor, which is responsible for differentiation of the first distal tubule segment. Reciprocally, embryos deficient in irx3b exhibited downregulation of hnf1ba/b transcripts in the distal early segment, suggesting a segment-specific regulatory circuit. Deficiency of hnf1ba/b also led to ectopic expansion of podocytes into the proximal tubule domain. Epistasis experiments showed that the formation of podocytes required wt1a, which encodes the Wilms’ tumor suppressor-1 transcription factor, and rbpj, which encodes a mediator of canonical Notch signaling, downstream or parallel to hnf1ba/b. Taken together, these results suggest that Hnf1β factors are essential for normal segmentation of nephrons during kidney organogenesis.


How renal progenitors differentiate into the different nephron segments is poorly understood. The zebrafish pronephric kidney has emerged as a useful model to study nephrogenesis due to the high degree of cellular and genetic conservation with the mammalian nephron but without a dependency on prior renal structures.1 The zebrafish pronephros comprises a single midline renal corpuscle attached to two tubules that are segmented into proximal and distal segments, including the proximal convoluted tubule (PCT), proximal straight tubule (PST), distal early tubule (DE), and distal late tubule (DL).2

The zebrafish pronephros arises from the intermediate mesoderm (IM) during development and initially expresses the transcription factor genes pax2a, pax8, and lhx1a.3–5 The anteriormost cells adopt a podocyte fate and express genes such as nephrin and podocin, encoding critical components of the glomerular blood filter.6 The formation of podocytes is dependent on the activity of the Wilms’ tumor suppressor-1a (Wt1a) transcription factor and Notch signaling, similar to observations in mammals.7–9 The remaining renal progenitors differentiate into a tubule with distinct proximal and distal segments. This process involves a considerable refinement in the expression domains of early expressed renal genes such as pax2a, and the activation of later-acting genes such as irx3b.
that is essential for the expression of DE segment-specific genes.10

In this study, we identified paralogues of *hepatocyte nuclear factor-1 beta* (hnf1ba and hnf1bb), encoding homeodomain transcription factors, as essential regulators of nephron formation. Zebrafish embryos deficient in hnf1ba/hnf1bb ectopically express podocyte markers in the region where the proximal tubule normally forms. Although an epithelial tubule arises from the remaining IM, it fails to express segment-specific solute transporter genes. Epistasis experiments revealed that hnf1ba/b acts upstream or in parallel to wta1 and Notch signaling to restrict podocyte fate. Taken together, these results implicate Hnf1β factors as key determinants of nephron segmentation that act to restrict podocyte formation and activate segment-specific gene expression programs.

**RESULTS**

**Hnf1b Factors Directly Regulate the cdh17 Promoter**

To better understand nephrogenesis, we developed a transgenic line in which enhanced green fluorescence protein (eGFP) was expressed in the pronephros. The *cadherin-17 (cdh17)* gene was chosen because it is highly expressed in all tubule segments.11 Although a 4.6 kb cdh17 promoter was initially identified,12 we subsequently found that 1.2 kb of genomic sequence upstream of the start codon is equally sufficient (Figure 1A). The spatiotemporal expression of this shorter promoter recapitulates the endogenous cdh17 gene expression pattern with eGFP fluorescence/transcripts being detected in all tubule segments from the 8-somite stage to 3 days postfertilization (dpf) (Supplemental Figure 1, A–C).

We performed a deletion mapping analysis to identify critical transcription factor binding sites in the cdh17 promoter (Supplemental Figure 2A). By transiently expressing these deletion constructs in embryos, a 300 bp region (−900 to −600 nucleotides) was identified as being essential for promoter activity (n=17 of 48 injected embryos were eGFP+; Figure 1A). This element contains sequence motifs similar to the consensus binding site for HNF1β, a homeodomain-containing transcription factor implicated in kidney development13 (Supplemental Figure 2B).

The zebrafish genome contains two Hnf1 paralogues, hnf1ba and hnf1bb.14 An analysis of their expression patterns showed that they are expressed in the developing pronephros from the 8-somite stage onward (Supplemental Figure 1, D and E), similar to previous reports.14–17 Whereas hnf1ba is expressed in all tubule segments, transcripts for hnf1bb are restricted to the proximal tubule segments and DE tubule segment with barely detectable levels in the DL segment (Supplemental Figure 1E). On the basis of these results, Hnf1ba and Hnf1bb were considered excellent candidates for regulating cdh17 expression. We examined the effect of deleting one of the putative Hnf1b binding sites (position −750) in the cdh17 promoter (Supplemental Figure 2B). This mutation severely compromised the activity of the cdh17 promoter (n=5 of 68 injected embryos were weakly eGFP+) compared with the intact promoter (n=36 of 64 injected embryos were eGFP+; Figure 1A) consistent with Hnf1β factors acting as direct inducers of cdh17.

**Nephron Expression of cdh17 is Lost in hnf1ba/b-Deficient Embryos**

We next investigated the effect of hnf1ba/b deficiency on cdh17 expression in vivo. Embryos homozygous for *h1843*, a recessive retrovirus-induced mutation in hnf1ba,17 develop pericardial edema and die around 5 dpf (Supplemental Figure 3A). Incrosses of hnf1ba/bh1843/+ fish resulted in a quarter of the offspring with reduced cdh17 expression in the DL segment (Figure 1B). We hypothesized that this restricted loss of cdh17 transcripts was due to redundant activities of hnf1bb in the other segments. Consistent with this, injection of an hnf1bb morpholino into hnf1ba/bh1843 homozygous embryos induced a loss of cdh17 expression from all segments (Figure 1B). Genotyping confirmed these embryos were homozygous for the hnf1ba/bh1843 allele (data not shown). No effect on cdh17 expression was seen with hnf1bb knockdown alone (Supplemental Figure 3B), suggesting that the activity of hnf1bb in the pronephros is redundant with hnf1ba. Taken together, these results confirm that Hnf1β factors regulate cdh17 expression in vivo.

**Nephron Segmentation, but not Epithelialization, Fails to Occur in hnf1ba/b-Deficient Embryos**

To assess the effect of hnf1ba/b deficiency on nephron segmentation, we examined a range of segment-restricted markers. We found that hnf1ba/b-deficient animals lack expression of all segment markers at 24 hours postfertilization (hpf) including *atp1a1a* (all segments), *slc12a3* (DE segment), *etv5a* and *trpm7* (PST segment), *slc12a1* and *kcnj1* (DE segment), *slc12a3* (DL segment), and *cldn* (DE and DL segments; Figure 2). In addition, transcripts for *odf3b* and *rfx2*, markers of multi-ciliated cells, are also absent in hnf1ba/b-deficient embryos (Figure 2). By contrast, relatively normal expression was found for the cloacal markers *gata3* and *aquaporin3a* (Figure 2).

To investigate if the pronephric tubules were apoptosing in hnf1ba/b-deficient embryos, we performed acridine orange staining but did not detect any significant cell death in the kidney (data not shown). Sectioning of hnf1ba/b-deficient and unaffected siblings stained for *atp1a1a* transcripts showed the presence of a tubule in the mutants, suggesting that epithelialization of the IM was not blocked (insets in Figure 2A). In support of this, we found expression of the epithelial markers *laminin5*, *cadherin1* (*cdh1*), and *epcam*, in the tubule of hnf1ba/b-deficient embryos (Figure 2B). A closer examination of the tubules by electron microscopy revealed small cuboidal epithelial cells with apical junctional complexes and a basement membrane. However, the brush border and tall morphology of the epithelial cells that characterize the PCT segment was absent and the tubule was collapsed with a
small lumen (Figure 3A). On the basis of these findings, we conclude that *hnf1ba* and *hnf1bb* are required to activate a nephron segmentation program in kidney progenitors independently of signals that induce their epithelialization.

### Early Acting Renal Regulators Are Not Downregulated in *hnf1ba/b*-Deficient Embryos

To better understand the basis of the nephron defects in *hnf1ba/b*-deficient embryos, we examined the expression of renal regulators *pax2a, pax8, lhx1a, and mecom.*\(^4,18–24\) Expression of *pax2a, pax8, and mecom* is lost from proximal portions of the pronephros after the 15-somite stage (excluding podocyte progenitors in the case of *pax2a*) but is retained in the DL segment.\(^4,10,19\) Expression of *lhx1a* is lost in all segments but retained in podocyte progenitors.\(^9,20\) In *hnf1ba/b*-deficient embryos, transcripts for *pax2a, pax8, lhx1a,* and *mecon* all fail to downregulate and instead persist in proximal regions of the pronephros at 24 hpf (Figure 3B). These findings indicate that the Hnf1β transcription factors regulate the spatiotemporal expression changes of *pax2a, pax8, mecom,* and *lhx1a* that occur before the appearance of mature nephron segments.

### Ectopic Podocyte Formation Occurs in *hnf1ba/b*-Deficient Embryos

In zebrafish, podocyte progenitors arise around the 12-somite stage and express *wt1a* and *wt1b,* orthologs of *Wt1.*\(^9,25,26\) These cells migrate to the midline and express podocyte genes such as *nephrin* and *podocin.*\(^6\) In *hnf1ba/b*-deficient embryos at 24 hpf, the expression domain of *wt1a* and *wt1b* was ectopically expanded into the region where the PCT segment normally
forms (Figure 4A). At 48 hpf, a similar expansion in nephrin
and podocin
 cells was observed in hnf1ba/b-deficient embryos and these cells failed to fuse at the midline (Figure 4, A and B). To better determine the timing of this fate change, we examined hnf1ba/b embryos at the 15-somite stage and observed an increased number of wt1b
 cells, indicative of increased podocyte progenitor formation (Figure 4C, arrowhead). At this stage, expression of hnf1ba
 and wt1b
 is mutually exclusive in wild-type embryos, as revealed by double in situ hybridization staining (Figure 4D). Taken together, these results suggest that Hnf1b factors act in presumptive PCT progenitors to prevent these cells adopting a podocyte progenitor fate.

Hnf1b Factors Act Downstream of Retinoic Acid Signaling during Podocyte Formation

Retinoic acid (RA) signaling is needed for both podocyte and proximal tubule segment formation.2,10 We therefore asked whether the effects of hnf1ba/b deficiency on podocyte formation were dependent on RA. We blocked RA signaling in hnf1ba/b-deficient embryos from the early gastrula stage to the 15-somite stage using diethylaminobenzaldehyde (DEAB).27 As previously reported, this results in a loss of podocytes and tubules composed only of distal segments (Figure 4E and Wingert et al.2). We found an absence of wt1b
 podocytes at 24 hpf in DEAB-treated hnf1ba/b-deficient embryos (Figure 4E), suggesting that Hnf1b factors act downstream of RA. We also found that expression of the DL marker, slc12a3, was abrogated in DEAB-treated hnf1ba/b-deficient embryos consistent with a requirement for Hnf1B factors to promote tubule differentiation downstream of RA (Figure 4E).

Hnf1b Factors Act Upstream or Parallel to wt1a and Notch Signaling during Podocyte Formation

We previously demonstrated that the formation of podocyte progenitors is dependent on wt1a
 and rbpj, encoding a transcriptional mediator of the Notch pathway.9 To determine the epistatic relationship between hnf1ba/b and wt1a
 and rbpj, we used morpholinos to knockdown wt1a
 and rbpj in hnf1ba/b-deficient embryos. Embryos deficient in all of these factors failed to develop podocytes, indicating that the development of ectopic podocytes in hnf1ba/b-deficient animals is dependent on wt1a
 and Notch signaling (Figure 4F). We also found that the expression of jagged2a (jag2a), encoding a Notch ligand implicated in podocyte formation,9 also failed to downregulate in the proximal portion of the pronephros in hnf1ba/b-deficient animals (Figure 4G).
To explore whether Hnf1β factors can suppress podocyte formation when overexpressed, we injected wild-type embryos with synthetic hnf1ba mRNA. Although a dose of hnf1ba mRNA was chosen that rescued nephron formation in hnf1ba/b-deficient embryos (data not shown), we failed to observe a reduction in wt1a or wt1b podocytes in the injected embryos at 24 hpf (n=35 of 35 injected embryos with wt1a podocytes and n=31 of 31 injected embryos with wt1b podocytes; Figure 4H). This suggests that forced expression of Hnf1b factors in the ‘normal’ podocyte domain is not sufficient to block podocyte specification.

Mammalian nephrons develop from pretubular aggregates that develop into renal vesicles and then comma- and S-shaped bodies with a proximal-distal axis.28 Proximal cells in these structures express Wt1 and are believed to contribute to podocytes, whereas the other portions likely give rise to tubule segments.29 In E15.5 mouse kidneys, we found transcripts for Hnf1b in the distal, but not proximal, domain of comma-shaped bodies (Figure 5A). Similarly, in S-shaped bodies, Hnf1b and Wt1 showed nonoverlapping expression domains (Figure 5A). These findings are consistent with a conserved role for Hnf1β factors in regulating the boundary between podocyte and tubule progenitors.

**Pax2a and Pax8 Are Required for hnf1ba/b Expression in the Nephron**

The pax2a and pax8 genes are expressed before the initiation of hnf1ba/b in the IM, making them excellent candidates as upstream inducers.9 To explore this, we examined embryos deficient in pax2a and pax8 by injecting pax2a morpholinos into embryos from a pax8<sup>pax8<sup>tm1<sup>cre</sup></sup> incres (where the pax8 gene has been disrupted by the DSRen gene). In approximately a quarter of the injected embryos, representing presumptive pax2a/pax8 doubly deficient animals, we observed a severe reduction of transcripts for both hnf1ba (25.5%; n=51) and hnf1bb (21.9%; n=41) at 24 hpf (Figure 5B). No effect on hnf1ba expression was seen in embryos singly deficient in either pax2a or pax8 (data not shown). These observations indicate that pax2a and pax8 are required for expression of hnf1ba/b in the pronephros.

If pax2a/pax8 acts upstream of hnf1ba/b, then we would expect nephrogenesis to be disrupted in pax2a/pax8-deficient animals to a similar extent as in hnf1ba/b-deficient animals. To confirm this, we examined pax2a/pax8 deficiency on the expression of mature segment markers and found a severe downregulation of cdt17, scl20a1a (PCT segment), slc12a1 (DE segment), and slc12a3 (DL segment) in presumptive pax2a/pax8-deficient embryos (Figure 5B).

**Irx3b-Deficient Embryos Display Downregulated Expression of hnf1ba in the DE Segment**

We previously showed that knockdown of irx3b results in defective DE segment differentiation with a loss of scl12a1 and kcnj11 expression.10 Given that the DE segment also fails to form in hnf1ba/b-deficient embryos, we investigated the epistatic relationships between hnf1ba/b and irx3b. In hnf1ba/b-deficient embryos at 24 hpf, irx3b was not expressed, consistent with the loss of the DE segment in these animals (Figure 6A). We next investigated whether irx3b was required to maintain hnf1ba expression in the DE segment. Wild-type embryos were injected with irx3b morpholinos and expression of hnf1ba was examined at 24 hpf. We found that transcripts for hnf1ba were reduced in the DE segment of the majority of irx3b-deficient embryos (82.6%; n=46), together with cdt17 (78%; n=41), atp1a1a4 (67%; n=39) and clcnk (100%; n=48; Figure 6B). These data suggest that Hnf1β factors are initially required to induce irx3b but irx3b is then needed at later stages to maintain hnf1ba expression in the DE segment.

**DISCUSSION**

Hnf1β, and the closely related factor Hnf1α, have emerged as important regulators of epithelial differentiation in multiple...
In the developing mouse kidney, Hnf1b is expressed in the ureteric bud, which branches into the collecting duct system, as well as the tubules.\(^{38,39}\) The role of Hnf1b in the kidney has been challenging to study because its constitutive inactivation leads to early lethality.\(^{38}\) A number of studies using different approaches to overcome this have implicated Hnf1b in ureteric bud growth/branching\(^{16,32,40}\) and the regulation of genes involved in cystic kidney disease.\(^{14,32,41,42}\) Although HNF1b mutations in humans support a link to cyst formation, they are also associated with severe congenital abnormalities of the kidney, suggestive of an early role of HNF1b in nephrogenesis.\(^{40,43}\) Experiments in frogs have further implicated Hnf1b factors in nephron formation but determining their precise function has been elusive.\(^{36,44,45}\) Here, using the zebrafish...
model, we demonstrate that Hnf1b factors act downstream of pax2a and pax8 to restrict podocyte fate and to establish the proximo-distal segmentation pattern of the nephron (Figure 7). In addition, evidence for transcriptional regulatory loops was identified between hnf1ba/b, pax2a, pax8, and irx3b.

Our finding that a tubular epithelium still forms in hnf1ba/b-deficient animals suggests that the mesenchymal-to-epithelial transition (MET) of the IM is an independent process from the activation of segment genes, such as solute transporters. Although we cannot rule out the possibility that defective maturation of the tubular epithelium contributes to the segmentation failure, we favor a model whereby Hnf1b factors act as key inducers of nephron differentiation in parallel to MET-inducing factors. Likely candidates for regulating MET are pax2a and pax8, as the IM in Pax2/8 double-mutant mouse embryos fails to undergo MET and is lost by apoptosis.22 In zebrafish, pax2a and pax8 are expressed before the hnf1b factors and pax2a/8-deficient embryos show decreased levels of hnf1ba and hnf1bb. Thus, we propose that Pax2a and Pax8 initiate nephron formation by performing two key functions: (1) inducing the MET of the IM and (2) inducing hnf1ba and hnf1bb expression.

Whereas the absence of the DE segment in hnf1ba/b-deficient animals can be explained by a failure to induce irx3b, the molecular basis for the absence of the other segments is unclear. One possibility is that Hnf1β drives expression of all segment-specific genes but only specific targets are activated due to the presence of unique combinations of transcriptional cofactors, repressors, or epigenetic modifications. In support of Hnf1β factors being “master tubule regulators,” other studies have identified target genes in both proximal and distal segments including FXYD2 (encoding the γ-subunit of Na’K’ ATPase),46 OAT1/slc22a6,47 OAT4/slc22a11,48 and URAT1/slc22a12.49 In addition, a recent bioinformatics approach identified potential Hnf1 binding sites in a large number of tubule genes, with a predominance in proximal tubule-restricted genes.50

RA is required for IM cells to adopt a podocyte fate and important targets include wt1a51 and the Notch ligand jag2a.2 These genes are not only expressed by podocyte progenitors, which arise from cells adjacent to somite 3, but also by a subset of PCT progenitors.2,9 These observations suggest that a mechanism exists to prevent Wt1a and Notch signaling from inducing a podocyte program in PCT progenitors. We propose that the Hnf1b factors confer this suppressive activity given that they act downstream of RA but upstream, or in parallel, to Wt1a and the Notch pathway to restrict podocyte

Figure 5. Mouse Hnf1b is expressed in distal portions of S-shaped bodies and pax2a/pax8-doubly deficient embryos have reduced expression of nephron segment markers. (A) In E15.5 mice, Hnf1b is distally restricted in comma-shaped bodies (left panel) and Hnf1b and Wt1 show nonoverlapping expression domains in S-shaped bodies (right panels). (B) pax8<sup>rfp/rfp</sup>/pax2a<sup>amo</sup> embryos show reduced expression of hnf1ba, hnf1bb, cdh17, slc20a1a, slc12a1, and slc12a3 at 24 hpf.

Figure 6. Irx3b regulates hnf1ba expression in the DE segment. (A) hnf1ba/b-deficient embryos do not express irx3b in the central region of the pronephric tubules. (B) irx3b-deficient embryos are created by morpholino knockdown. In these embryos, we observe reduced hnf1ba, cdh17, atp1a1a.4, and clcnk expression in the DE segment (arrowheads).
formation. This function is in agreement with the expression pattern of *hnf1ba*, which is non-overlapping with *wt1b* podocyte progenitors at the 15-somite stage. In developing mouse nephrons, mutually exclusive expression domains for *Wt1* and *Hnf1b* are also found, raising the possibility that the regulation of podocyte specification by *Hnf1b* is conserved in mammals.

Simple overexpression of *hnf1ba* was not sufficient to inhibit podocyte specification and further work is needed to understand how Hnf1b factors regulate the podocyte/tubule boundary.

In summary, we have demonstrated new roles for the Hnf1b factors as major regulators of nephron segmentation and podocyte formation. These functions appear to act independently to pathways that promote epithelial tubulogenesis, strongly suggesting that Hnf1b factors function to induce a renal-specific program within a "generic" epithelium. These findings provide novel insights into the functions of Hnf1b during nephrogenesis and will help guide future studies into the causes of kidney cysts and renal birth defects in patients with HNF1b mutations.

**CONCISE METHODS**

**Zebrafish Husbandry**

Zebrafish were maintained and staged according to established procedures. Embryos were collected from paired matings of *hnf1ba* heterozygous, *pax8* heterozygous, or wild-type Tubingen adults.

**Genotyping**

*Hnf1bahi1843* fish were isolated from a retroviral insertional mutagenesis screen and are considered to represent a null allele due to insertion of the vector into exon 2 of the *hnf1ba* gene. To identify embryos carrying the retroviral insert (homozygous or heterozygous), we extracted genomic DNA from individual embryos using a lysis buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl2, 0.3% Tween20, 0.3% NP40) and proteinase K (10 mg/ml). Embryos were incubated overnight in the lysis buffer at 55°C, and then incubated at 98°C for 10 minutes to kill the proteinase K. Primers specific to the *LacZ* gene present in the vector were used to amplify a 364 bp product from this embryo genomic DNA (forward: 5'-ATCCTCTAGACTGCCATGG-3'; reverse: 5'-ATCGTAACCGTGCATCTG-3'). To distinguish homozygotes from heterozygotes, primers flanking the vector insertion site were used that generated a 271 bp product when the wild-type allele was present (forward: 5'-AATTCAACCAGGCCACAC-3'; reverse: 5'-CGGTTGCACTCCTCCACC-3'). We used the following cycling conditions: 10 seconds at 98°C denaturation step, 15 seconds at 72°C extension step for 25 cycles, and a final 10 minutes 72°C extension using the Finnzymes Phusion hot start DNA polymerase.

**Morpholinos and mRNA Synthesis**

Previously validated morpholinos to *wt1a* (5'-CACGAACATCAGAACC-CATTTTGAG-3'26), *rbpja/b* (5'-CAAACTTCCCTGTCACAACGGCGC-3'53), *pax2a* (5-TATGTGCTTTTCTTACCTTCCGAG-3'54) *hnf1bb* (5'-CTTGGACACCATGTCAGTAAA-3'15), and *irx3b* (5'-ACCGGGAGGACTCGGGGAACTCG-3'10) were purchased.

All animal husbandry adhered to the accredited Code of Animal Conduct and was approved by the University of Auckland Animal Ethics Committee.

**Promoter Analyses of the cdh17 Transgene Design**

A *cdh17;GFP* transgenic line that drove GFP expression in the pronephros, yolk, and gut was initially made using a 4.6 kb genomic fragment upstream of the first exon of the *cdh17* locus. We further refined this promoter to a 1.2 kb fragment of 5′ upstream sequence and used this construct to undertake a deletion analysis by PCR and subcloning. A consensus HNF1 site at position −750 was deleted and replaced with a FseI restriction enzyme site by PCR and subcloning. Promoter constructs in the pTol2 transposase vector were injected into one-cell stage embryos together with Tol2 transposase mRNA and imaged at later stages using a Nikon 80i compound microscope and a Hamamatsu ORCA camera. Some of these embryos were raised to adulthood and stable transgenic lines expressing GFP were generated to confirm the results from the transient injections.

**Figure 7.** A model for the spatiotemporal regulation of nephron segmentation by Hnf1b factors in zebrafish.
sections were acetylated, dehydrated, and hybridized with 500 ng/ml 4% PFA in PBS for 10 minutes and treated with 10 ng/ml RNase A at 37°C for 15 minutes, and incubated with anti-Digoxigenin-AP (1:4000) at 4°C overnight. Sections were incubated with BM purple and color reaction was terminated by fixation in 4% PFA in PBS. Slides were mounted with the Glycergel mounting media.

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**DISCLOSURES**

None.

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