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NRAGE: A potential rheostat during branching morphogenesis

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ABSTRACT

Branching morphogenesis is a developmental process characteristic of many organ systems. Specifically, during renal branching morphogenesis, its been postulated that the final number of nephrons formed is one key clinical factor in the development of hypertension in adulthood. As it has been established that BMPs regulate, in part, renal activity of p38 MAP kinase ($p38^{MAPK}$) and it has demonstrated that the cytoplasmic protein Neurotrophin Receptor MAGE homologue (NRAGE) augments $p38^{MAPK}$ activation, it was hypothesized that a decrease in the expression of NRAGE during renal branching would result in decreased branching of the UB that correlated with changes in $p38^{MAPK}$ activation. To verify this, the expression of NRAGE was reduced in *ex vivo* kidney explants cultures using antisense morpholino. Morpholino treated *ex vivo* kidney explants expression were severely stunted in branching, a trait that was rescued with the addition of exogenous GDNF. Renal explants also demonstrated a precipitous drop in $p38^{MAPK}$ activation that too was reversed in the presence of recombinant GDNF. RNA profiling of NRAGE diminished *ex vivo* kidney explants resulted in altered expression of GDNF, Ret, BMP7 and BMPRIb mRNAs. Our results suggested that in early kidney development NRAGE might have multiple roles during renal branching morphogenesis through association with both the BMP and GDNF signaling pathways.

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1. Introduction

Essential hypertension, or hypertension with no identifiable cause, is unfortunately a common disease of the Western world (Kearney et al., 2005). In the early 1970's David Barker proposed the "fetal origins of disease hypothesis", supposing that the prevalence of many adult diseases, including hypertension, is a result of abnormal fetal development (Barker et al., 1970). Brenner later refined this hypothesis by proposing that lower nephron numbers predisposed individuals to essential hypertension (Brenner et al., 1988). Since reports based on the Brenner–Barker hypothesis suggest a link between kidney development and hypertension (Langley and Jackson, 1994; Levitt et al., 1996; Woodall et al., 1996), elucidating the molecular mechanisms that govern kidney development could elucidate the key factors affecting the development of hypertension later in life.

The development of the kidney begins with renal branching morphogenesis (RBM). During RBM reciprocal inductive interactions, between the ureteric bud (UB) and the surrounding

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metanephric mesenchyme (MM) result in the development of the collecting ducts and the nephrons. The precise molecular signals that control RBM are currently unknown and still actively pursued. Bone morphogenic proteins (BMPs) are members of the transforming growth factor beta (TGFβ) superfamily of signaling molecules and have been implicated in a diverse array of biological processes, including cell growth, differentiation and apoptosis (Hogan, 1996). BMPs play crucial roles during RBM, transducing their signal either through the canonical SMAD-mediated pathway, and/or through the non-canonical BMP signaling cascade of MAP kinases, TAK1, TAB1, and $p38^{MAPK}$ (Nohe et al., 2004; Oxburgh et al., 2004; Winnier et al., 1995).

We recently demonstrated that NRAGE is a potential member of the non-canonical BMP pathway utilizing the multipotential neural progenitor cells resulting in BMP instructive apoptosis (Kendall et al., 2005). It has been suggested that the same non-canonical BMP signaling pathway also mediates branching of the UB (Hu et al., 2004) suggesting a potential role for NRAGE during embryonic renal branching morphogenesis. It was hypothesized that a decrease in the expression of NRAGE during RBM would result in altered branching of the UB and potentially in cell viability.

Utilizing NRAGE morpholinos (Kendall et al., 2005), we attenuated NRAGE protein expression in *ex vivo* kidney culture explants to determine if decreased NRAGE expression affects $p38^{MAPK}$ activation and consequently branching of the UB. We also investigated the global ramifications of lowering NRAGE expression in the developing explants in hopes of elucidating other pathways and mechanisms that NRAGE may regulate during renal development. As predicted, lowering NRAGE expression severely retarded the growth and branching of the UB. What was surprising and unexpected was that gene profiling revealed that lowering NRAGE levels lead to a reduction in the expression of BMPR1b, Ret, GDNF, and BMP7 in the developing kidney. Rescue experiments demonstrated that exogenously applied recombinant GDNF corrected the deficiency in branching in *ex vivo* explants cultures, with GDNF being more robust to promote growth and branching than BMP7. These results demonstrate the importance of NRAGE in affecting the maximal response during branching morphogenesis.

2. Methods

2.1. Cell culture

mIMCD-3 (ATCC, Virginia, USA) cells were cultured in DMEM/F12 (Invitrogen, California, USA) supplemented with 10% fetal bovine serum (Hyclone, Utah, USA) in a 37 °C and 5% CO_2 humidified incubator. In branching experiments, 1×10^6 cells were plated in a collagen matrix as described by Piscione without modification (Piscione et al., 2001). The matrix was assembled on ice and plated with various doses of GDNF (0–10 ng/ml) (R&D Systems, Minnesota, USA), BMP7 (0–10 ng/ml) (R&D Systems, Minnesota, USA), no supplementation, or TGFβ (0–25 ng/ml) (R&D Systems, Minnesota, USA) for 3–14 days with media refreshed every morning.

2.2. Co-immunoprecipitation and immunoblotting

Cell lysates were generated from mIMCD-3 cells that were treated with and without 10 ng/ml BMP7 (R&D Systems, Minnesota, USA) for 1 h in DMEM-F12 (Invitrogen, California, USA). Cells were lysed in 350 μl of NPB lysis buffer consisting of: 20 mM Tris, pH 7.5, containing 300 mM sucrose, 60 mM KCl, 15 mM NaCl, 5% (v/v) glycerol, 2 mM EDTA, 1% (v/v) Triton X-100, with protease inhibitor cocktail I (Sigma–Aldrich, Missouri, USA) for 20 min on ice. Lysates were immunoprecipitated using 50 μl of G-sepharose beads (Amersham Biosciences–GE Healthcare, USA) and 2 μg of NRAGE (1:1000) or Ret antibody (1:2000) (Upstate-Millipore, Massacheusetts, USA; R&D Systems, Minnesota, USA) overnight at 4 °C. The beads were collected by centrifugation at 12,000 RPMs for 5 min and washed three times with fresh ice-cold lysis buffer. The samples were subjected to 12% SDS–PAGE under reducing conditions. After transferring the resolved proteins to Hybond C membrane (Amersham Biosciences–GE Healthcare, USA), blots were probed for NRAGE (1:1000) (Upstate-Millipore, Massachusetts, USA), TAK1 (1:1000) (Upstate-Millipore, Massachusetts, USA), Tab1 (1:1000) (Pro-Sci, California, USA), XIAP (1:1000) (Cell Signaling Technology, Massachusetts, USA) or β-actin (1:2500) (Sigma–Aldrich, Missouri, USA) antibodies. Blots were developed using an appropriate horseradish peroxidase conjugated goat anti-mouse or rabbit IgG (Bio-Rad) and the ECL detection system (Amersham Biosciences–GE Healthcare, USA).

2.3. Kidney organ culture

Kidney organ culture was performed as previously described by Nikopoulos et al. (2008) using kidneys from E11.5 Hoxb7-GFP mice (Srinivas et al., 1999) or E11.5 ICR mouse embryos (Taconic, New York, USA). Morpholino sequences used in this study are as follows, NRAGE morpholino: GGTTTCTGAGCCATAGCTCTCGTC and for the negative control morpholino: CCTCTTACCTCAGTTACAATTTATA (GeneTools, Oregon, USA). BMP7 or GDNF (R&D Systems, Minnesota, USA) was added at concentrations and time described for each experiment. Kidneys were analyzed under a Leica stereomicroscope (Leica, USA) or subjected to immunofluorescent staining using: TOPRO-3 (1:10000; Invitrogen, California, USA) to identify nuclei, or Ki67 (1:1000; AbCam, Massachusetts, USA) to identify proliferating cells, Dolichous Bifluorous Agglutinin to identify cells of the ureteric bud (DBA; 1:1000; Vector Labs, California, USA) or anti-laminin to also identify cells of the ureteric bud (Sigma–Aldrich, Missouri, USA). Kidney explants stained with Ki67 were visualized using a Leica TCS-SP confocal microscope (Leica, USA). The number of Ki67 positive cells was determined by counting the Ki67 positive nuclei in a given field for each kidney analyzed and calculating the number of cells per μm^3 using the Leica TCS software.

2.4. TUNEL analysis

E11.5 Hoxb7-GFP kidney explants were cultured with either NRAGE morpholino or negative control for 72 h, in DMEM/F12 media + 10% FBS prior to being in 4% PFA over-