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The *lmx1b* gene is pivotal in glomus development in *Xenopus laevis*

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ABSTRACT

We have previously shown that *lmx1b*, a LIM homeodomain protein, is expressed in the pronephric glomus. We now show temporal and spatial expression patterns of *lmx1b* and its potential binding partners in both dissected pronephric anlagen and in individual dissected components of stage 42 pronephroi. Morpholino oligonucleotide knock-down of *lmx1b* establishes a role for *lmx1b* in the development of the pronephric components. Depletion of lmx1b results in the formation of a glomus with reduced size. Pronephric tubules were also shown to be reduced in structure and/or coiling whereas more distal tubule structure was unaffected. Over-expression of *lmx1b* mRNA resulted in no significant phenotype. Given that lmx1b protein is known to function as a heterodimer, we have over-expressed *lmx1b* mRNA alone or in combination with potential interacting molecules and analysed the effects on kidney structures. Phenotypes observed by over-expression of *lim1* and *ldb1* are partially rescued by co-injection with *lmx1b* mRNA. Animal cap experiments confirm that co-injection of *lmx1b* with potential binding partners can up-regulate pronephric molecular markers suggesting that *lmx1b* lies upstream of *wt1* in the gene network controlling glomus differentiation. This places *lmx1b* in a genetic hierarchy involved in pronephros development and suggests that it is the balance in levels of binding partners together with restricted expression domains of *lmx1b* and *lim1* which influences differentiation into glomus or tubule derivatives *in vivo*.

Introduction

The amphibian pronephros is derived from the intermediate mesoderm and consists of a single non-integrated nephron. The vascularised filtration unit, the glomus, forms from blood vessels branching from the dorsal aorta, and is not directly connected to the tubule but forms on one of the sides of the body cavity known as the nephrocoel, while the tubule anlage forms on the other (Drummond and Majumdar, 2003). The proximal tubules and glomus are specified by stage 12.5, and the more distal tubule components are specified between stages 13 and 14 (Brennan et al., 1998, 1999). At stage 21, the pronephric anlage is indicated by a thickening of the lateral mesoderm, the differentiation of the kidney compartments starts at stage 28. At stage 31, the first nephrostome begins to function and the entire pronephros is fully functional by stage 37/38 with the Wolffian duct developed (Nieuwkoop and Faber, 1956). Since the pronephros is such a simple organ, it is an ideal model for the study of the later kidney forms, as many of the genes and pathways known to play a role in later kidney development are also expressed in the pronephros, where they are presumed to have an equally important role (Vize et al., 1997). Although there is increasing knowledge of the molecular control of pronephrogenesis, much of the molecular basis of induction and patterning of the pronephric glomus is still poorly understood (Saxén, 1987; Brändli, 1999; Drummond and Majumdar, 2003; Jones, 2003; Vize, 2003a).

LIM domain proteins have been shown to have roles in the development of the kidney (reviewed in Jones, 2003). *lim1* is expressed in the organiser and the notochord during gastrulation and then in the intermediate mesoderm of the presumptive pronephros at tailbud stages (Taira et al., 1992, 1994) and is often used as a molecular marker of pronephric tubules. Co-injection of *lim1* with *pax8* (a DNA-binding transcription factor) mRNA produces abnormally large pronephroi and ectopic tubules indicating a major role in kidney differentiation (Carroll and Vize, 1999). Recently it has been suggested that *lim1* does not initiate differentiation of the pronephros but that it is necessary for growth and elongation in the development of the pronephric tubules (Chan et al., 2000). The mouse homolog *lim1* also appears to play an important role in kidney development as a *lim1* knock-out results in embryos that lack pronephros, mesonephros and metanephros (Shawlot and Behringer, 1995).

Abbreviations: lmx1b, Xenopus LIM homeobox 1b; NPS, Nail Patella Syndrome; MO1 and MO2, lmx1b morpholinos; MO, Morpholino.

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In the last few years, we have identified another member of the LIM homeodomain family in *Xenopus laevis lmx1b*[4], which is highly expressed in the pronephric glomus in a temporal expression pattern consistent with a major role in glomus development (Haldin et al., 2003). *Lmx1b* is also expressed in the kidney of other vertebrates. In chick embryos, *c-lmx-1* transcripts have been detected in the mesonephros (Riddle et al., 1995) and a recent study demonstrates the exclusive expression of *Lmx1b* in the mouse glomerulus (Suleiman et al., 2007).

The *LMX1b* gene maps to the Nail Patella Syndrome (NPS) locus in humans (Dreyer et al., 1998). NPS is an autosomal dominant condition, characterised by skeletal abnormalities, nail defects and nephropathy (Knoers et al., 2000; Sweeney et al., 2003). The main renal pathology is a defect in the glomerular basement membrane, associated with abnormal podocytes, leading, in extreme cases, to renal failure and death. Pathogenetic mutations of *LMX1b* have been identified most of which lie in the codons encoding the LIM and homeodomains domains but some have also been described in the COOH-terminal third of the protein (Dreyer et al., 1998; McIntosh et al., 1998; Clough et al., 1999; Vollrath et al., 1998; Dunston et al., 2004; Millá et al., 2007). Conventional mutants of the mouse *Lmx1b* gene, which is 99% identical to human LMX1B, show skeletal and renal phenotypes very similar to those seen in NPS patients (Chen et al., 1998; Miner et al., 2002; Rohr et al., 2002). Mutant kidneys are characterised by distended convoluted tubules with accumulation of glycoproteins, prominent thickening of the glomerular basement membrane with occasional regions of membrane discontinuity and podocyte malformation. Unfortunately, homozygous mutant animals die 24 h after birth, preventing any further analysis during the development of the metanephric components. The creation of another *Lmx1b* transgenic model, a constitutive podocyte-specific knock-out mouse, demonstrates the role of *Lmx1b* in the initial differentiation and also in the maintenance of podocytes (Suleiman et al., 2007).

Several proteins have been identified as binding partners for LIM homeodomain proteins, mediating the transcriptional activities of LIM domain proteins. Ldb1 (or Clim2) can bind to LIM domains and synergize with lim1 *in vivo* (Agulnick et al., 1996). Ldb1 has also been shown to interact with Lmx1b (Marini et al., 2003) and to regulate its transcriptional activity (Dreyer et al., 2000). Moreover, Lmx1b has been shown to cooperate with the basic helix–loop–helix protein E47/shPan in activating promoter activity (German et al., 1992; Dreyer et al., 2000), an activation that can be down-regulated by Ldb1 (Dreyer et al., 2000).

We report here the functional roles of *lmx1b* and its potential binding partners in pronephrogenesis, together with a detailed study of their distribution in dissected pronephric anlagen in the model vertebrate *Xenopus laevis*. Knock-down of *lmx1b* by morpholino (MO) technology, using two independent morpholinos, prevented full development of the glomus but also affected formation of the pronephric tubules. We demonstrate that both of these phenotypes can be rescued by over-expression of *lmx1b-mut* mRNA which fails to bind to both MO sequences, but still encodes wild type protein. Over-expression of *lmx1b* alone resulted in no significant pronephric phenotype. However, over-expression of *lmx1b* with its potential binding partners *ldb1* and *lim1* had significant effects on kidney structures. Over-expression of *lim1* induced enlargement of both glomus and tubules whereas over-expression of *ldb1*, in contrast, caused a reduction in size of all pronephric components. Co-injection of *lmx1b* with either *lim1* or *ldb1* partially rescues these phenotypes. Cap experiments with co-injected mRNAs provide direct evidence for the role of LIM domain proteins in the up-regulation of pronephric genes, such as *pax2*, *pax8* and *wt1* but also differentiated podocyte markers such as *podocin* and *podocalyxin*. This is the first study to document the role of these important molecules in vertebrate pronephric development and we propose a model for the roles of the interactions between both *lmx1b* and *ldb1*, and *lim1* and *ldb1* during glomus and tubule development respectively in *Xenopus*.

Materials and methods

Production, dissection and microinjection of Xenopus laevis embryos

Embryos were produced by *in vitro* fertilisation by standard procedures, dejellied in 2% cysteine–HCL pH8.0, and washed several times with 1/10 BarthX (BarthX is 0.1 M NaCl, 2 mM KCl, 10 mM $MgSO_4$, 2 mM $CaCl_2$, 5 mM Hepes, 0.1 mM EDTA). Embryos were then cultured to the required stage in 1/10 Barth X and 10 mg/ml gentamycin. Staging was according to Nieuwkoop and Faber (1994).

The intermediate mesoderm, pronephric anlagen and developing pronephros were dissected with an eyebrow hair knife as described in Brennan et al. (1998). The pronephric proximal/intermediate tubules formed the major part of the dissected tissues from embryos at stages 35/36 and 37/38, due to the difficulty of dissecting the other more distal pronephric tubule components.

The different components of the pronephros, glomus, proximal and intermediate tubules, distal and connecting tubules (see Reggiani et al., 2007) were removed from one side of 40 MS222 (ethyl-*n*-aminobenzoate methane sulfonate acid; tricaine methanesulfonate) anaesthetised embryos at stage 42. The epidermis was removed with an eyebrow hair knife and the whole pronephros teased out. The glomus was separated and the distal and connecting tubules dissected away from the proximal tubule mass. The intermediate tubules, which cannot be separated easily by eye, were included in the proximal tubule sample.

Animal caps were dissected in BarthX from stage 8/9 injected embryos using forceps and an eyebrow hair knife and cultured in BarthX until stage 26. Animal caps and control embryos were then harvested and RT-PCR performed.

Microinjections were performed at the one cell stage, one cell of 2-cell embryos or into the V2 blastomere of 8-cell stage embryos. The V2 injections effectively target mRNAs to the somitic and pronephric lineages (Huang et al., 1998; Moody and Kline, 1990). β-galactosidase (LacZ) or GFP (2 ng) was co-injected as lineage tracer. Red-Gal or X-Gal staining to identify the injected side was carried out as described in Kyuno et al., 2003.

Morpholinos

lmx1b morpholino 1 (MO1) (5′-tgcaatatccatgccactctccaaa-3′) and morpholino 2 (MO2) (5′-gccactctccaaaactcacttcagt-3′) were designed and supplied by GeneTools, LLC. MO's (5 ng/nl) were injected (5 to 20 ng) alone or in combination with mRNA (2.5 ng). The random sequence control morpholino (cMO) (5′-cctcttacctcagttacaatttata-3′) designed by GeneTools was used. *anxa4a* MO1 (Seville et al., 2002) was also used to control the specificity of the *lmx1b* MO.

Expression clones, mRNA synthesis and microinjection

Capped mRNAs were transcribed *in vitro*, from template DNA previously linearised with the appropriate restriction enzymes with mMessage Machine™ kits from Ambion (SP6 or T3 RNA polymerase). mRNA synthesis was as follows; *lmx1b*:pRN3, SfiI and T3 RNA polymerase; *lmx1b-mut*:pRN3, SfiI and T3 RNA polymerase; *lmx1b*:pCS3+MT, EcoRI and RNA SP6 polymerase; *BJ069617* and B*J038889*, XhoI and T3 RNA polymerase; *ldb-1*:pSP64RI, SalI and SP6 RNA polymerase; *lim1*:pSP64T, SalI and SP6 RNA polymerase; *E47p*:pGEM-T Easy, SstII and SP6 RNA polymerase; *wt1*:pSP64TS, SstI and Sp6 RNA polymerase; *anxa4a*:pRN3, SfiI and T3 RNA polymerase (Seville et al., 2002).

[4] GenBank Accession no. AF414086.

In vitro and in vivo translation of construct mRNA

mRNA (0.5 μg) was translated *in vitro* in the Rabbit Reticulocyte Lysate System (Promega) according to manufacturer's protocol in presence of 10 μCi of [^{35}S] Methionine alone or with MO (10 μg). Translation products were analysed by autoradiography (Kodak) after an overnight exposure of the 10% SDS-polyacrylamide gel.

For *in vivo* analysis, 25 ng of mRNA was microinjected into oocytes. After 24 h, oocytes were homogenised into 50 μl of homogenisation buffer (150 mM NaCl, 0.1% NP-40, 50 mM Tris–HCl pH8, 1 mM PMSF) and proteins were extracted following centrifugation.

Western blot

Protein samples were run on a 10% SDS-PAGE gel and electrophoretically transferred onto nitrocellulose membrane (Amersham) using a Mini Trans-Blot Electophoretic Transfer cell (Biorad). C-myc tagged proteins were detected using an anti-myc monoclonal antibody 9E10 antibody (Sigma) and a rabbit anti-mouse HRP-conjugated secondary antibody (Sigma) following standard protocol.

Rt-pcr

Total RNA from whole or dissected embryos was extracted, and cDNA synthesis and non radioactive RT-PCR were performed as described by Barnett et al. (1998). Primers and amplification conditions used in this work are listed in Table 1. Each experiment contained −RNA, −RT and −cDNA negative controls and a linearity series to show that the PCR was in the linear range. For each experiment, the quantity of input cDNA was determined by equalisation of the ODC (ornithine decarboxylase) *or* EF1α *signal.*

Table 1
Primer sequences and PCR conditions for the required molecular markers

Marker size (bp)	Primer sequence (5′–3′)	Annealing temp. °C	No. cycles	Reference
lmx1b 291	U-GGAGAGTGGCATGGATATTG D-AGTAGCAGCTGGTGGTGAGG	60	28	Haldin et al. (2003)
globin/lmx1b 310	U-GCAGAAGCTCAGAATAAACGC D-AGTAGCAGCTGGTGGTGAGG	60	27	Haldin, Thesis
lim1 444	U-GAAGGATGAGACCACTGGTGG D-CACTGCCGTTTCGTTCATTTC	60	28–29	Witta and Sato (1997)
ldb1 391	U-CTAGCCATGCATGCCCAAGAC D-GTTCGCCCACCACCATTACATG	57	30	This work
E47 428	U-GGCTTTCCTGCTGCTGTGCTCT D-TCTCGTACTCGCACTCTTTCTCGT	55	30	Simrick, thesis
wt1 436	U-CACACGCACGGGGTCT D-TGCATGTTGTGATGACG	55	29–32	Carroll and Vize (1996)
clck 183	U-TCACTGCGTCCGACGACTCT D-TCTATGCTGCACTCCTCACC	55	31	This work
nephrin 536	U- GCAGCACCGCCTATAACTTCTCAG D-CATATCCGCCCTCTTCCCAACCTT	55	32	This work
Na,K-ATPase subunit gamma 279	U-GCCTCATTCAGATGCCTCTT D-TGCTGGTGCAGTCAGTGTAA	55	32	This work
pax-2 250	U-TCGGAAGAAGAGTGGTCTAC D-GGTATTCATATTCCGCATTC	55	30	Haldin, thesis
pax-8 276	U-CCAACAGCAGCATCAGATC D-CAATGACACCTGGCCGGATA	53	28	Haldin et al. (2003)
pod1 384	U-TCTCAGTGATGTGGAGGACTTC D-TGACGCAGGTGAGCTATGTAAC	57	34	Simrick et al. (2005)
podocin 500	U-AGAGGACCTGGTCTCTTCTT D-CAGATAGTTTTTCAGCAGCCA	59	31	This work
podocalyxin 295	U-CCAGAGATGCAGGAGAAAAA D-CAAACCAGACGTATCAAAAGAA	55	32	Simrick, thesis
SMP30 262	U-TTAGACTGGTCTCTGGATCAC D-CGATAGGTAACTTTACAGTCTG	55	31	Sato et al. (2000)
ODC 131	U-GGAGCTGCAAGTTGGAGA D-TCAGTTGCCAGTGTGTGGTC	55	28	Bassez et al. (1990)
EF1α 270	U-CAGATTGGTGCTGGATATGC D-CACTGCCTTGATGACTCCTA	55	20	Mohun et al. (1989)

Wholemount in situ hybridisation

Wholemount *in situ* hybridisation was carried out using a standard protocol (Harland, 1991). Microinjected embryos were fixed in MEMFA (0.1 M MOPS pH 7.4, 100 mM EGTA, 1 mM MgSO4 and 4% formaldehyde) and hybridised with the antisense *wt1* or *nephrin* RNA probe. The *wt1* probe was transcribed *in vitro* with T3 RNA polymerase after linearization of wt1:pGEM7 by SacI and the *nephrin* probe was transcribed with T7 RNA polymerase from nephrin:pCMV-Sport6 previously linearized with SmaI. Probes were synthesised and labelled using a DIG labelling kit (Roche) and the hybridisation visualised using sheep anti-DIG-alkaline phosphatase antibody (Roche) and 4-nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolylphosphate substrate (NBT/BCIP; Roche). After fixation overnight in Bouin's fixative, non-specific staining was removed by several washes in 70% ethanol/PBS and embryos were bleached (1% H_2O_2, 5% formamide and 0.5× SSC) on a fluorescent light source.

Wholemount immunostaining

Wholemount double immunohistochemistry was performed on microinjected pigmented embryos fixed in MEMFA and dehydrated in methanol after being washed in water. Embryos were rehydrated in PBS, bleached and immunostained with monoclonal antibody 3G8, specific for pronephric proximal tubules, as described in Vize et al. (1995). The colour reaction was performed using NBT/BCIP (purple staining) (Roche). Samples were fixed in MEMFA for 1 h at 4 °C and the second round of immunohistochemistry was performed with 4A6, a monoclonal antibody specific for intermediate, distal and connecting tubules (Vize et al., 1995) using Fast Red TR/Naphthol AS-MX (red staining) (Sigma).

Wax embedding, sectioning and Haematoxylin/Eosin staining

Injected embryos were fixed in Bouin Picro Formol fixative (BDH Laboratory Supplies) for 2 h at room temperature. After intensive washing in 70% ethanol/PBS, the embryos were dehydrated gradually in absolute ethanol. Samples were embedded in wax and sectioned at 11 μm as described in Haldin et al. (2003). Rehydrated sections were then stained in Haematoxylin Harris (Surgipath 01562E) for 2 min, washed successively in water, acid alcohol (70% ethanol, 0.5% HCL), water and Scott's solution ($MgSO_4$ 20 g, $NaHCO_3$ 2 g, dH_2O 1l) before staining in 1% eosin (Sigma). Slides were finally washed under running water, dehydrated and after xylene washes, mounted using DePex mountant (BDH Laboratory Supplies).

Measurement of embryos and statistical analysis

Following *wt1* or *nephrin in situ* hybridisation, images of each group of embryos were captured at the same magnification using a Nikon SMZ1500 microscope and digital DXM1200F camera. The area of staining in the glomus of both sides of the embryos was measured using the computer software LuciaG. The area of the injected side was compared to the area of the uninjected contra-lateral side. Means, variance and standard deviations were calculated and *t*-test analysis was carried out. Chi-squared tests were carried out for each experiment analysed for tubule morphology following 3G8 and 4A6 immunostaining. Each experiment was performed in duplicate on separate batches of embryos.

Results

lmx1b and its potential binding partners display specific temporal expression patterns during pronephric development

We have analysed by semi-quantitative RT-PCR the temporal expression profiles in dissected kidney anlagen of *lmx1b* and its potential binding partners *lim1*, *ldb1* and *E47* in addition to other important genes expressed early in pronephrogenesis. This technique is more sensitive than *in situ* hybridisation and therefore can allow the detection of genes expressed at relatively low levels. We show that the two LIM domain genes *lim1* and *lmx1b* are expressed from stage 13 in the presumptive pronephric tissues and remain expressed at significant levels at all the stages tested. *ldb1* and *E47* are expressed at all stages tested. By stage 15 both *pax8* and *pod1* are transcriptionally activated in advance of the podocyte marker *nephrin* and *wt1*. *pax2* transcripts can be detected weakly from stage 13 consistent with low levels of *pax2* which can be detected in animal caps at these early stages (Fig. 1A and data not shown).

In addition, the different pronephric components were manually dissected from stage 42 tadpoles and the distribution of *lmx1b*, *lim1*, *ldb1* and *E47* assessed (Fig. 1B). The quality of the dissections was assessed with kidney specific markers: *nephrin* (Gerth et al., 2005) and *podocin* (Boute et al., 2000) for glomus, *pax8* (Heller and Brandli, 1999) and *SMP30* (Sato et al., 2000) for proximal tubules, *clck* (Vize, 2003b) and *Na,K-ATPase subunit gamma* for the tubule compartments (Eid and Brändli, 2001). *Wt1* is expressed in the glomus but also to a lesser extent in the other pronephric dissected components, due to unavoidable contamination of the dissections with coelomic epithelium. *lmx1b* is also expressed more in the glomus than in the different tubule fractions; this expression in the tubules compartments might be due to glomerular impurities since *nephrin*, *wt1* and *podocin* are also detected in these samples. *lim1* on the other hand is more expressed in the tubule fractions and is almost absent from the glomus. *ldb1* is expressed at constant levels in all pronephric components. *E47* is expressed at higher levels in the tubule fractions than in the glomus. This data demonstrates temporal and regional expression of LIM domain proteins and their potential binding partners in the pronephros suggesting that they play a specific role in pronephric development.

lmx1b morpholino prevents normal glomus development

Two distinct *lmx1b* morpholinos (MO1 and MO2) were designed to disrupt translation of lmx1b protein (Supplementary Fig. 1A). MO1 hybridisation spans the postulated start codon of *lmx1b* mRNA (Haldin et al., 2003) in addition to 13 bases in the 5′ UTR whereas MO2 is located in the 5′UTR sequences, upstream of the ATG. To confirm the specificity of the two MOs, *lmx1b* and/or *lmx1b-c-myc* mRNAs were translated in the presence or absence of the *lmx1b* MO in both an *in vitro* rabbit reticulocyte lysate system and *in vivo* in oocytes (Supplementary Fig. 1 panels B, C and D). Both available pseudoallelic genes were tested (Supplementary Fig. 2 panels A and B). These data confirm the specificity of both morpholinos for lmx1b knockdown experiments. Embryos injected at the 1 cell stage (data not shown) or into the V2 blastomere at the 8-cell stage with *lmx1b* MO1 or MO2 showed no toxic effects and were analysed by *in situ* hybridisation for the glomus specific *wt1* expression domain at stage 35/36. Fig. 2A, shows that depletion of lmx1b by MO1 (compare panel b to e) or MO2 (compare panel h to k) prevents development of a full size glomus on the injected side. The *wt1 in situ* domain in the cMO-injected embryos identified a large, oval-shaped domain of the glomus identical to the uninjected side (compare panels a to d and g to j,). Staining in an elongated area towards the posterior end of the embryo corresponding to hybridisation to the walls of the coelomic cavity can also be seen in some embryos dependant on how long the embryos were left in the colorimetric stage. Embryos injected with *lmx1b* MO1 and MO2 showed a highly reduced glomus. The oval shape of the glomus capsule was still evident in most embryos although reduced in

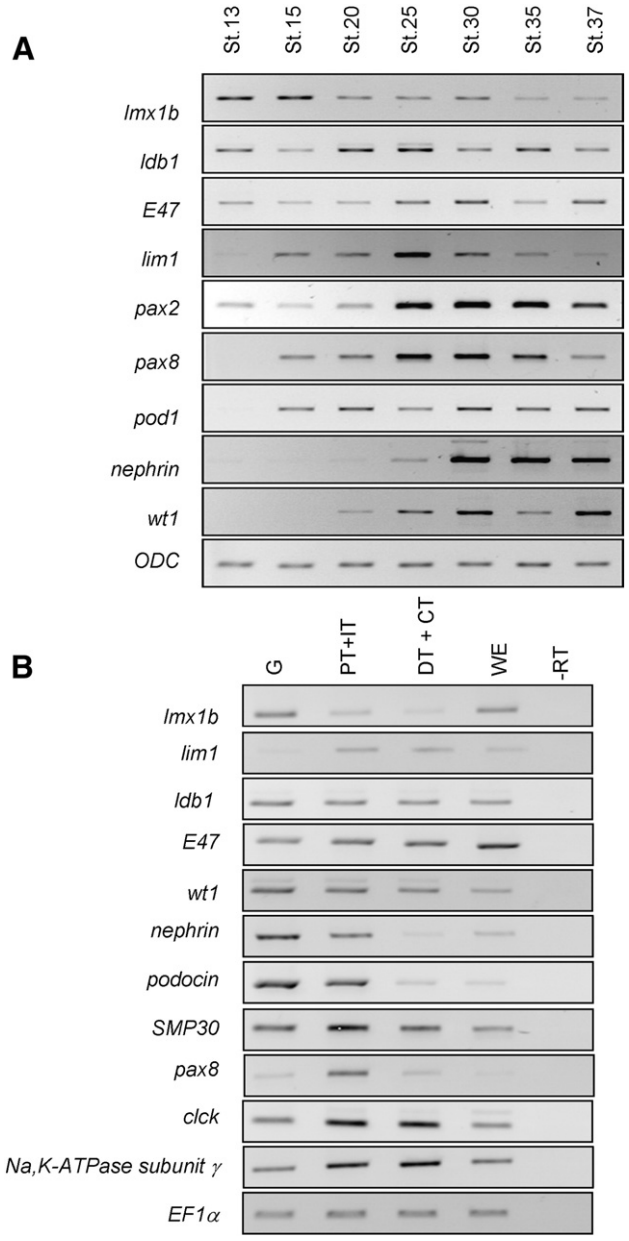

Fig. 1. lmx1b and its binding partners display different temporal and spatial pronephric expression. (A) Embryos at different stages were dissected to isolate the intermediate mesoderm from the presumptive pronephric region (stage 13), the pronephric anlagen (stages 15 and 20) and the pronephros (stages 25, 30, 35/36 and 37/38). RT-PCR analysis shows *lmx1b*, *ldb1*, *lim1* and *E47* are expressed from stage 13 in the presumptive pronephric tissue and their expression is maintained in the pronephros until stage 37/38. *Pax2* is also expressed from this early stage at a low level, whereas *pax8* and *pod1* transcripts are detected from stage 15. *Wt1* is only expressed from stage 20 in the pronephros. *ODC* was used as a loading control. (B) The different pronephric components were dissected from stage 42 embryos and the spatial expression of *lmx1b* and its binding partners was analysed by RT-PCR. Analysis shows that *ldb1* is expressed in all three components of the kidney at a similar level whereas *E47* is more expressed in the proximal and intermediate tubules and in the distal and connecting tubules. As expected, *lmx1b* transcripts are mostly detected in the glomus whereas *lim1* is expressed in the tubules. The identity of each dissected components was verified using specific pronephric markers: glomus by *nephrin* and *podocin*; proximal and intermediate tubules by *pax8* and *SMP30*; as expected *clck* is mostly expressed in the distal and connecting tubules and *Na,K-ATPase subunit gamma* is detected in tubule components whereas *wt1* is more expressed in the glomus. *EF1*α was used as a loading control. G: Glomus; PT: Proximal tubules; IT: Intermediate tubules; DT: distal tubules; CT: Connecting Tubules; WE: Whole Embryo.

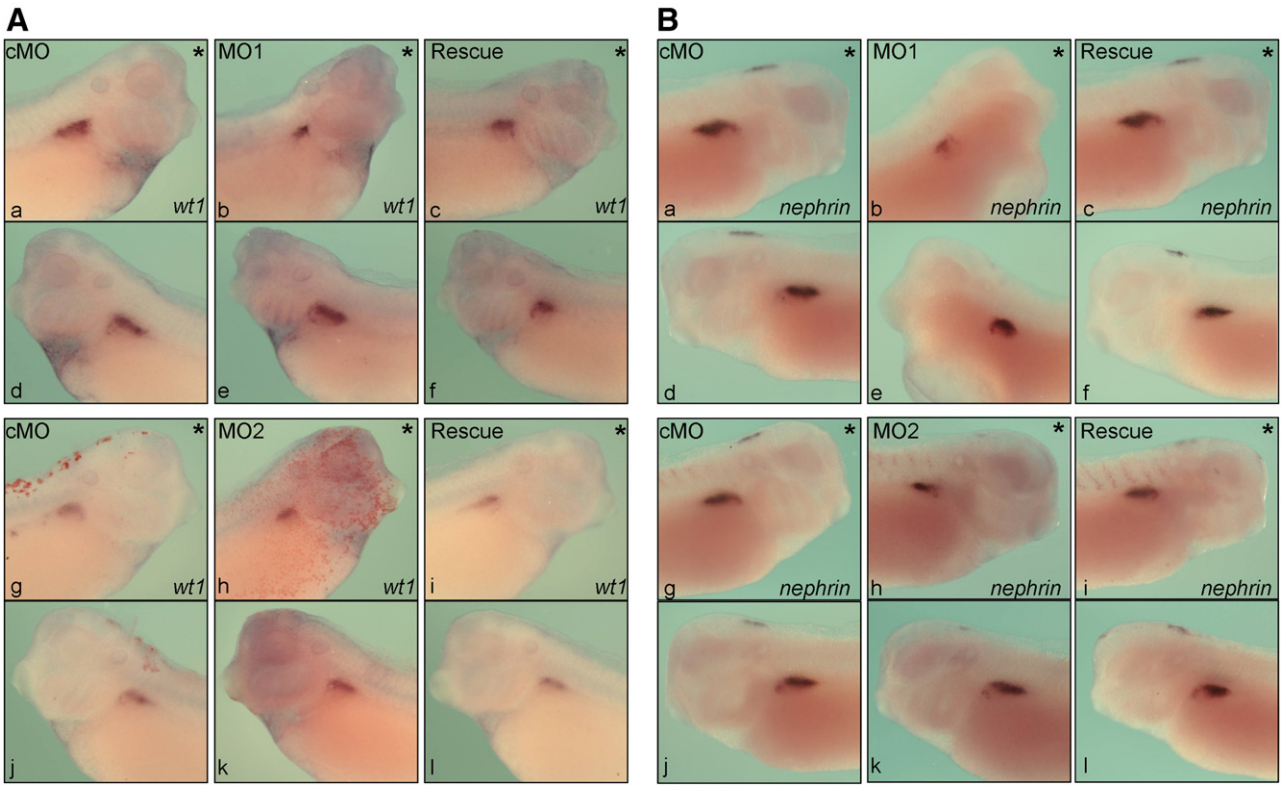

Fig. 2. *lmx1b* MO1 and MO2 prevent full size glomus formation. (A) *lmx1b* MO1 (panels b, e) or MO2 (panels h and k) was injected into the V2 blastomere at the 8-cell stage. All injections were carried out using a lineage tracer, either GFP (panels a–f) or LacZ (panels g–l). The size of the glomus was assessed by *wt1 in situ* hybridisation at stage 35/36. Injection of cMO does not induce any phenotype on the injected side (compare panels a to d and g to j). *lmx1b* MO1 injected embryos (5 ng) showed a reduced glomus (compare panel b to e). A similar phenotype was observed following the injection of *lmx1b* MO2 (10 ng) (compare panels h to k). Injection of *lmx1b-mut* mRNA (2.5 ng) can partially rescue the phenotype of MO1 (panels c and f) and MO2 (panels i and l). The injected side is marked with an asterisk. (B) *lmx1b* MO1 (panels b, e) or MO2 (panels h and k) was injected into one cell of 2-cell embryos. All injections were done using a lineage tracer, either GFP (panels a–f) or LacZ (panels g–l). The size of the glomus was assessed by *nephrin in situ* hybridisation at stage 33/34. Injection of cMO does not induce any phenotype on the injected side (compare panels a to d and g to j). *lmx1b* MO1 injected embryos (5 ng) showed a reduced nephrin-staining area on the injected side (compare panels b to e). A similar phenotype was observed following the injection of *lmx1b* MO2 (10 ng) (compare panels h to k). Injection of *lmx1b-mut* mRNA (2.5 ng) can partially rescue the phenotype of MO1 (panels c and f) and MO2 (panels i and l). The injected side is marked with an asterisk.

size and sometimes abnormal in shape. The expression of *wt1* in the walls of the coelomic cavity was totally eliminated. Injection of *lmx1b* MO at the one cell stage sometimes caused some additional perturbation of normal development (data not shown) consistent with the expression domains of *lmx1b* in the nervous system, resulting in a slightly shortened anterior/posterior axis (Haldin et al., 2003).

In order to establish the statistical significance of the reduction of glomus size, the area of *wt1 in situ* staining in the glomus region was measured on both sides of the embryo to generate a numerical value of glomus area using the Lucia G image analysis. This allowed full statistical comparison using the paired *t*-test between injected and uninjected sides (Supplementary Table 1). All comparisons were made using embryos of the same batch, injected on the same day. No phenotype can be observed after injection of cMO whereas injection of *lmx1b* MO1 (5 ng) or MO2 (10 ng) induced consistently significant reduction in the relative area of glomus staining on the injected side (the two tailed value *P* was 0.0038 and 0.0007 respectively). The expression of *wt1* was reduced by 32% and 34% on the injected side following injection of lmx1b MO1 and MO2 respectively.

To confirm that the effect on glomus development was specific, *lmx1b* MO1 or MO2 was co-injected with approximately 2.5 ng of *lmx1b-mut* mRNA whose translation is unaffected in the presence of MO1 and MO2 (Supplementary Figs. 1B, C). Embryos were cultured until stage 35/36 and the effects on glomus morphology was analysed by *wt1 in situ* hybridisation. In both cases, embryos co-injected with MO1 or MO2 and *lmx1b-mut* mRNA had significantly larger glomus structures than MO alone treated embryos on the injected side (compare panel c to b and i to h) and no significant differences can be seen between injected and uninjected sides (compare panel c to f and i to l) (Supplementary Table 1).

To confirm that the results obtained by *in situ* analysis were actually due to a smaller glomus rather than just loss of the *wt1* marker, MO injected embryos were analysed by *in situ* hybridisation for *nephrin*, an additional glomus marker at stage 33/34 (Fig. 2B). Similar phenotypes were observed. The *nephrin* domain was smaller on the injected side following knock-down of lmx1b by MO1 (compare panel b, e) or by MO2 (compare panel h, k). Following the injection of 10 ng MO2, the *nephrin* domain was reduced by 42% on the injected side. This phenotype was rescued by the injection of 2.5 ng of *lmx1b-mut* RNA (panel c, f and i, l). No differences in staining area could be observed between the injected and uninjected side and the embryos showed normal glomus on both sides, as seen in cMO-injected embryos (panels a, d and g, j).

Undifferentiated or damaged podocytes could lead to the loss of podocyte molecular marker expression. To assess if the reduction in staining by whole mount *in situ* analysis was due to a smaller glomus or to damaged podocytes, MO injected embryos were analysed by histological analyses. Transverse wax sections of *lmx1b* MO2 or cMO-injected embryos were Haematoxylin/Eosin stained and morphology of the pronephros analysed (Fig. 3). *Lmx1b* MO2-injected embryos display smaller glomus or lack any glomus structure on the injected side (panel a) compared to the uninjected side (panel b). No morphological differences could be observed after injection of cMO (panels c and d). In some cases, pronephric tubules were also reduced on the injected side of MO2-injected embryos (data not shown). Taken altogether, these data demonstrate that depletion of lmx1b prevents

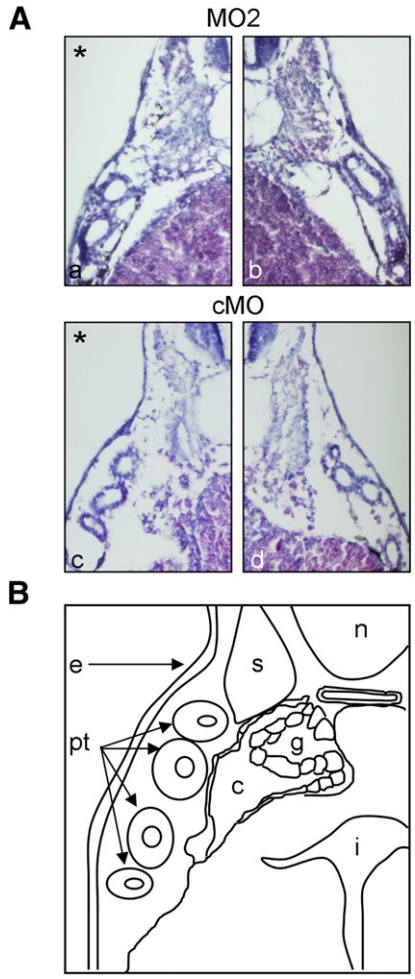

Fig. 3. *lmx1b* MO2 prevent full size glomus formation. (A) *lmx1b* MO2 (20 ng) (panels a and b) or cMO (20 ng) (panels c and d) was injected into the V2 blastomere at the 8-cell stage. All injections were carried out using GFP as a lineage tracer and embryos were selected based on the fluorescence before being fixed at stage 39. Wax transverse sections were stained with Hematoxylin and Eosin. Embryos injected with *lmx1b* MO2 display lack of or reduced glomus on the injected side (panel a) whereas injection of cMO do not affect the formation of the glomus (panel c). The injected side is marked with an asterisk. (B) Schematic drawing of a transverse section showing the structures of the glomus and pronephric tubules. c: coelome; e: ectoderm; g: glomus; i: intestine; n: notochord; s: somites; pt: pronephric tubules. Redrawn from H. Field (1891, Fig. 47).

formation of the glomus in *Xenopus*, rather than just preventing glomus differentiation marker expression.

lmx1b morpholino knock-down reduces pronephric tubule development

We also assessed whether MO depletion of lmx1b could affect the development of the pronephric tubules. 1-cell embryos were injected with either 10–20 ng of *lmx1b* MO1 or cMO and incubated until approximately stage 40. Immunohistochemistry was then carried out using the monoclonal antibodies 3G8 and 4A6, markers of proximal tubules and intermediate/distal/connecting tubules respectively (Vize et al., 1995). Knock-down of lmx1b affects tubule formation. A basic scoring system was devised where embryos were scored as either with normal, reduced, or very reduced tubules (see Supplementary Table 2 for full data). The scoring system took into account both a reduction in the total amount of proximal tubules identified by the 3G8 staining, and also the amount of coiling observed. Both pronephroi were scored for each injected embryo. MO1 treated embryos show the reduced tubule phenotype in 80% of embryos, the remaining being normal (Fig. 4A–b, Supplementary Table 2A). No phenotype could be observed in cMO-injected embryos (Fig. 4A compare a to d). Statistical analysis using Chi-squared was performed using the null hypothesis "there is no difference in pronephric tubule phenotype between *lmx1b* MO1 and cMO-injected embryos". The results confirmed that the depletion of lmx1b expression produced a significant difference at the 99% confidence level, disproving the null hypothesis (Supplementary Table 2B). Targeted injections into the V2 blastomere at the 8-cell stage yielded the same significant phenotype (Fig. 4B). Knock-down of lmx1b by MO1 (Fig. 4B, compare f to h) or by MO2 (Fig. 4B, compare b to d) induces formation of a significant reduction of proximal tubules on the injected side.

Like the glomus phenotype, the tubule phenotype could be rescued by over-expression of *lmx1b-mut* mRNA in embryos injected at the 1 cell stage (Fig. 4A–c and Supplementary Table 2). 35.5% of the embryos display normal tubule morphology (compared to 10.9% MO1 injected embryos) and only 22.2% show very reduced tubules (compared to 43.9% of MO1 injected embryos). The statistical significance of the rescue was demonstrated by Chi-squared analysis using the null hypothesis "co-injection of *lmx1b* MO1 and *lmx1b-mut* mRNA produces the same tubules phenotype observed in *lmx1b* MO1 injected embryos" at the 99% confidence level (Supplementary Table 2B). The tubule phenotype induced by knock-down of lmx1b using MO2 in V2 targeted embryos could also be rescued by injection of

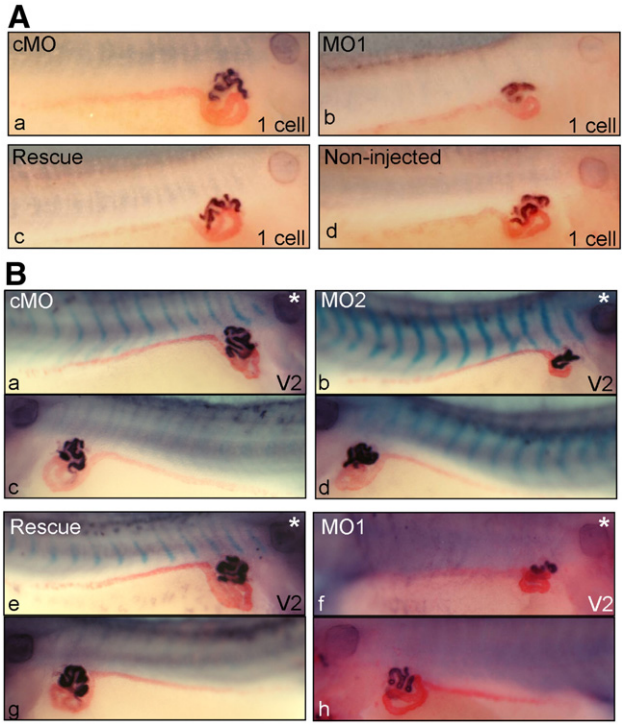

Fig. 4. *lmx1b* MO affects development of the pronephric tubules. (A) *lmx1b* MO1 was injected into one-cell embryos. Morphology of the pronephric tubules was assessed by immunohistochemistry using 3G8 (in purple) and 4A6 (in red) antibodies at stage 40 (Vize et al., 1995). Injection of the control MO (20 ng) had no effect on the formation of the pronephric tubules (a) and embryos displayed normal pronephros morphology as compared to control non-injected embryos (d). Injection of *lmx1b* MO1 (20 ng) affected the development of the proximal tubules, whereas the formation of the more distal tubules are unaffected (compare panel b to a). MO1-injected embryos showed reduced or very reduced proximal tubules. This phenotype can be rescued with the injection of *lmx1b-mut* mRNA (3 ng) (panel c). (B) control MO (panels a, c), *lmx1b* MO2 (panels b, d) or MO1 (panels f, h) and MO2 and *lmx1b-mut* mRNA (panels e, g) were injected into the V2 blastomere at 8-cells stage. LacZ (panels a–e, g) or GFP (panels f, h) was injected as lineage tracer. Injection of cMO has no effect on pronephric development (compare panel a to c) whereas knock-down of lmx1b by MO2 affected the development of the proximal tubules (compare panel b to d) on the injected side, as seen after injection of MO1 (compare panel f to h). The phenotype can be rescued with the over-expression of *lmx1b-mut* mRNA (panels e and g).

lmx1b-mut mRNA (Fig. 4B, compare e to b), resulting in the formation of normal pronephric proximal tubules on the injected side.

No significant effect was observed on the morphology of the more posterior tubules components stained by 4A6 (Fig. 4). Intermediate, distal and connecting tubules were only affected on embryos which displayed a severe reduction of the proximal pronephric tubules which was considered a secondary effect (data not shown).

Over-expression of lmx1b mRNA and its potential binding partners affects glomus formation

Since the LIM-HD proteins identified in other species exclusively exert their effects in combination with specific binding partners, we adopted a whole embryo assay to determine whether over-expression of potential binding partners and *lmx1b* either alone or in combination could generate a pronephric phenotype. This approach allowed us to modulate levels of potentially interacting binding partners.

We adopted a targeted approach to limit the toxicity affects of mRNA over-expression and enhance the phenotypic effects specifically in the pronephric region. 3 ng of *lmx1b*, *lim1*, *ldb1* or *E47* mRNA's were injected alone or in combination together with *LacZ* mRNA as a lineage tracer into one V2 blastomere at the 8-cell stage. As a control, lineage tracer alone was also injected. Only embryos that showed lineage tracer in the somites and pronephric region and which were phenotypically normal in gross morphology were analysed. No kidney phenotype was induced by control lineage injected embryos (Supplementary Tables 3–5).

To confirm that exogenous mRNA persisted in injected embryos up to and including the stages where specification and patterning of the kidney occurs, RT-PCR was carried out with primers specific for the injected mRNA and not endogenous *lmx1b* mRNA (Table 1). Injected mRNA transcripts could be detected in abundance up until stage 22 and were still present at lower levels until stage 35/36, which is considerably past the time of kidney specification and glomus formation (Supplementary Fig. 3).

Potential effects on glomus formation induced by lmx1b and its binding partners were assessed by *in situ* hybridisation for two glomus markers, *wt1* and *nephrin*. *Lmx1b* mRNA over-expression has no effect on the *wt1* expression pattern, whereas over-expression of its potential binding partners *ldb1* and *lim1* alone resulted in opposite phenotypes; a statistically significant reduction in glomus area ($p<0.0001$) or a significant glomus enlargement ($p=0.0022$) respectively (Fig. 5A compare a–c to e–g and Supplementary Table 3A). Co-injection of *lmx1b* with *ldb1* completely rescued the *ldb1* phenotype, significantly increasing glomus structure to a normal size (Fig. 5 compare B–c to A–c), so that no significant difference could be observed between the injected and uninjected sides (Fig. 5B, c and g; Supplementary Table 3A). Co-injection of *lmx1b* with *lim1* could partially rescue the *lim1* phenotype, by decreasing the glomus to a more normal size (Fig. 5; compare B–b to A–b; Supplementary Table 3A). Co-injection of *ldb1* and *lim1* mRNAs rescued both *ldb1* and *lim1*

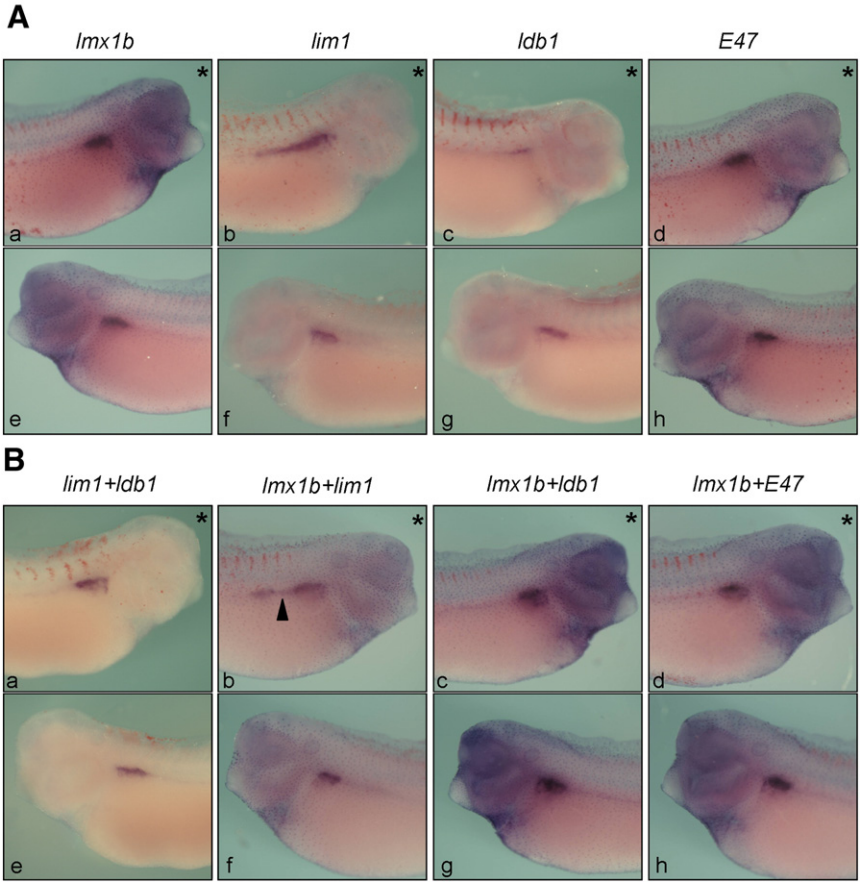

Fig. 5. Over-expression of *lmx1b* and its potential binding partners affects *wt1* expression. mRNAs of *lmx1b* and its potential binding partners were injected either alone (panel A) or in combination (panel B), together with *lacZ*, into one V2 blastomere at the 8-cell stage. Red Gal staining followed by *wt1 in situ* hybridisation was carried out at stage 35/36 in order to assess any glomus phenotype. For each embryo, the injected side (indicated by an asterisk, panels a–d) was compared to the uninjected side (panels e–h). Injection of *lmx1b* mRNA alone did not induce any significant glomus phenotype (A, compare panel a to e), whereas injection of *lim1* or *ldb1* alone, its potential binding partners, resulted in the formation of an enlarged and reduced glomus respectively (A, b and f; A, c and g). These phenotypes can be partially rescued by the co-injection of *lmx1b* (B, b, arrowhead and f; B, c and g). Interestingly, co-injection of *lim1* and *ldb1* rescued both phenotypes, resulting in the formation of a more normal glomus (B, a and e). Injection of *E47* alone or in combination with *lmx1b* did not induce any statistically significant phenotype (A, d and h and B, d and h).

phenotype since no significant differences could be seen between injected and uninjected sides (Fig. 5B compare a to e; Supplementary Table 3A). Injection of *E47* either alone or in combination with *lmx1b* had no effect on glomus structure (Figs. 5A–d and B–d; Supplementary Table 3A).

The analysis carried out with a specific *nephrin* antisense probe confirmed these phenotypes. *Lmx1b* or *E47* mRNA did not induce any significant phenotypes whereas injection of *lim1* and *ldpb1* resulted in a significant increase ($p=0.0471$) or decrease ($p<0.0001$) of *nephrin* stained domain respectively (Fig. 6A, Supplementary Table 3B). Co-injection of *lmx1b* and *lim1* rescued the *lim1* phenotype (Figs. 6 compare B–b to A–b, Supplementary Table 3B). Co-injection of *lmx1b* or *lim1* with *ldb1* could partially rescue the *ldb1* phenotype, increasing the *nephrin* stained domain to a more normal size (Figs. 6, compare B–c and B–a to 6A–c, Supplementary Table 3B). In this experiment, co-injection of *E47* and *lmx1b* slightly reduced *nephrin* expression on the injected side.

Over-expression of lmx1b mRNA and its potential binding partners affects the development of the other pronephric components

The effect of over-expression of these potential binding partner combinations on tubule structure was also assessed by immunohistochemistry with 3G8 and 4A6 antibodies (Fig. 7).

Over-expression of *lmx1b* alone failed to have any phenotype on any tubule domains (Fig. 7A compare a to e, Supplementary Tables 4 and 5). However, *lim1* alone caused an anterior/posterior enlargement of the proximal tubule domain with tubules underlying approximately 5–6 somite widths (Fig. 7A compare b and f). We confirmed the observation of Carroll and Vize (1999) that formation of smaller tubules could also be observed in some embryos (Supplementary Table 4A). Defects on the morphology of more distal tubules elements were also induced by *lim1* over-expression (Fig. 7A–b and Supplementary Table 5A). Injection of *ldb1* mRNA alone caused the opposite phenotype with a statistically significant reduction in size of tubule domain (Fig. 7A compare c to g and Supplementary Table 4).

Co-injection of *lmx1b* and *lim1* partially rescued the *lim1* over-expression phenotypes, increasing the size of the most posterior tubules to a more normal morphology and decreasing the percentage of reduced proximal tubules (Fig. 7B–b Supplementary Tables 4A and 5A). However, statistical analysis by the Chi-squared test showed that the rescue effect was significant only on more distal tubule morphology as labelled by 4A6 antibody (Supplementary Tables 4B and 5B). Co-injection of *lmx1b* with *ldb1* also partially rescued the *ldb1* tubule phenotype, with almost 43% embryos displaying normal proximal tubule morphology (Fig. 7B–c and Supplementary Table 4A). The reduction in more distal tubules size caused by over-expression of *ldb1* alone was worsened by the addition of *lmx1b* (Supplementary Table 5A). The Chi-squared test was used to compare the phenotype resulting from over-expression of both messages with that of *ldb1* over-expression alone. The null hypothesis set was "there is no difference in tubules morphology between *ldb1* and *lmx1b* and *ldb1* injected embryos". A significant difference was observed in the case of

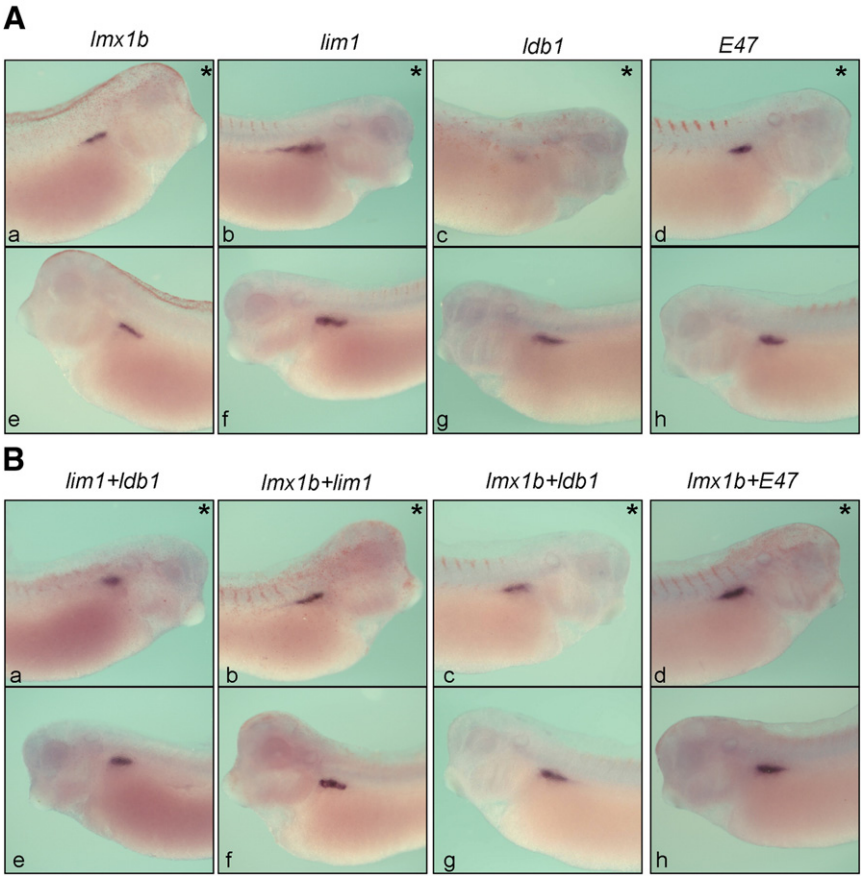

Fig. 6. Over-expression of *lmx1b* and its potential binding partners affects *nephrin* expression. mRNAs of *lmx1b* and its potential binding partners were injected either alone (panel A) or in combination (panel B), together with *lacZ*, into one cell of 2-cell embryos. Red Gal staining followed by *nephrin in situ* hybridisation was carried out at stage 33/34 in order to assess any glomus phenotype. For each embryo, the injected side (indicated by an asterisk, panels a–d) was compared to the uninjected side (panels e–h). Injection of *lmx1b* mRNA alone did not induce any significant change in *nephrin* staining (A, compare panel a to e), whereas injection of *lim1* or *ldb1* alone resulted in the formation of an enlarged and reduced *nephrin* domain respectively (A, b and f; A, c and g). These phenotypes can be partially rescued by the co-injection of *lmx1b* (B, b and f; B, c and g). Co-injection of *lim1* and *ldb1* rescued both phenotypes, the embryos displayed on the injected side a *nephrin* domain, similar in area to the uninjected side (B, a and e). Injection of *E47* alone or in combination with *lmx1b* did not induce any statistically significant phenotype (A, d and h and B, d and h).

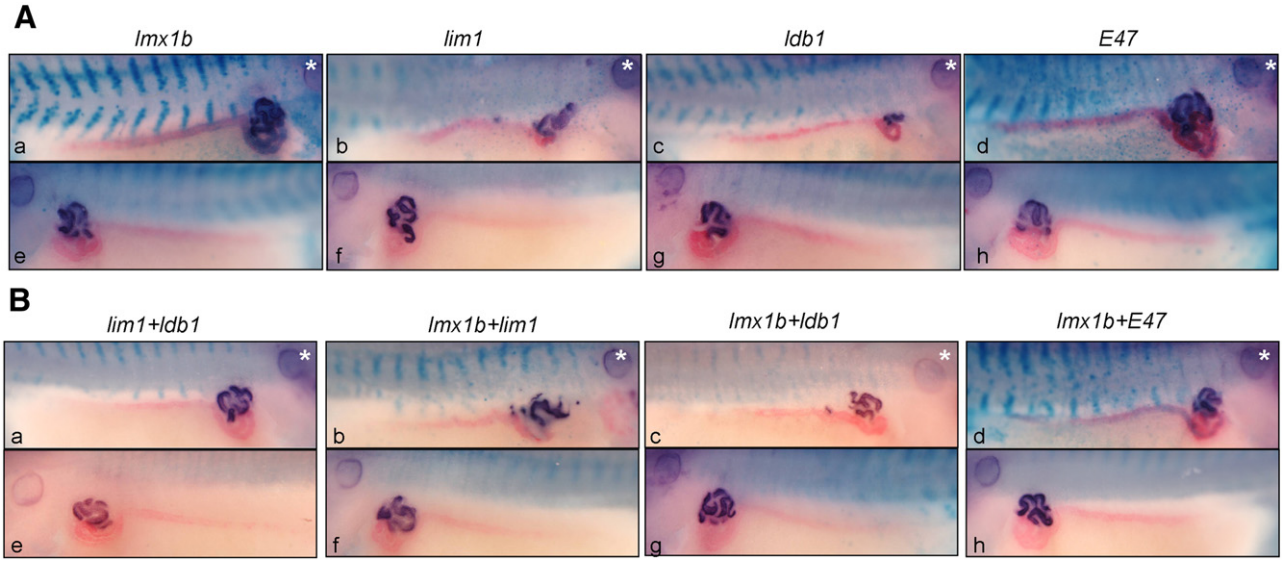

Fig. 7. Over-expression of *lmx1b* and its potential binding partners affects the development of tubules. mRNAs of *lmx1b* and its potential binding partners were injected either alone (panel A) or in combination (panel B), together with *lacZ* into one V2 blastomere at the 8-cell stage. The morphology of the tubules was assessed at stage 40 by immunohistochemistry using 3G8 (in purple) and 4A6 (in red) monoclonal antibodies (Vize et al., 1995). The injected side was identified by blue β-galactosidase staining and is indicated by an asterisk (a–d). As comparison, the uninjected side of each embryo was photographed (e–h) *lmx1b*-injected embryos showed no pronephric phenotype (A–a and e). *lim1* injection resulted in the formation of an enlarged proximal tubule mass and wider more distal tubules (A–b and f), whereas *ldb1* over-expression caused reduction in size of proximal tubules and in some cases affected formation of the more distal tubules (A–c and g). Co-injection of *lmx1b* with either *lim1* or *ldb1* partially rescued these phenotypes (B–b and f and B–c and g). Co-expression of *lim1* and *ldb1* partially rescued both *lim1* and *ldb1* phenotypes (B–a and e). Injection of *E47* resulted in the formation of slightly enlarged proximal tubule mass without affecting the more distal tubules (A–d and h) whereas co-injection of *lmx1b* and *E47* caused the opposite effect with reduction of pronephric proximal tubules (B–d and h).

intermediate/distal/connecting tubules analysis at the 95% confidence level (Supplementary Tables 4B and 5B).

Over-expression of *lim1* and *ldb1* resulted in phenotypes on tubules which were intermediate between those caused by the injection of each of the single mRNA's alone (Fig. 7B–a; Supplementary Tables 4A and 5A). The Chi-squared tests showed that changes observed in proximal tubule morphology were significant, suggesting that a synergy existed between these two proteins during formation of the most proximal tubules (Supplementary Tables 4B and 5B).

Over-expression of *E47* alone showed a statistically significant enlargement of the proximal tubule mass but unlike *lim1* over-expression, the mass remained in its normal A/P position (Fig. 7A–d). This phenotype was rescued by co-expression with *lmx1b* (Fig. 7B–d; Supplementary Tables 4). No effect on more posterior tubule morphology could be seen after injection of *E47* alone or in combination with *lmx1b* (Supplementary Tables 5).

Over-expression of lmx1b with potential binding partners in animal caps modulates kidney marker gene expression

One-cell embryos were injected with 3 ng of *lmx1b*, *lim1*, *ldb1*, *E47* and *wt1* alone or in combination with *lmx1b*. Animal caps were taken at stage 8/9 and incubated until stage 26, according to control sibling whole embryos. RT-PCR analysis shows that *lmx1b* over-expression alone could induce the expression of *pax2/8* and *podocalyxin* (Fig. 8). This effect could be mediated by the endogenous expression of *ldb1* in normal caps, allowing interaction with exogenous *lmx1b* mRNA which might induce expression of kidney markers since over-expression of lmx1b and ldb1 up-regulate the same profile of genes. However, all the double injection combinations up-regulate the expression of *pax2* and *pax8*. The combination of *lmx1b* and *lim1* up-regulates the expression of *wt1*, *podocalyxin* and *podocin* all differentiation markers of podocytes indicating formation of glomus tissue. *Podocalyxin* is also up-regulated to a lesser extent by *lmx1b* and *ldb1* and *lmx1b* and *wt1*, relative to control caps. No up-regulation of *nephrin* expression was induced in this experiment, suggesting that the expression of nephrin

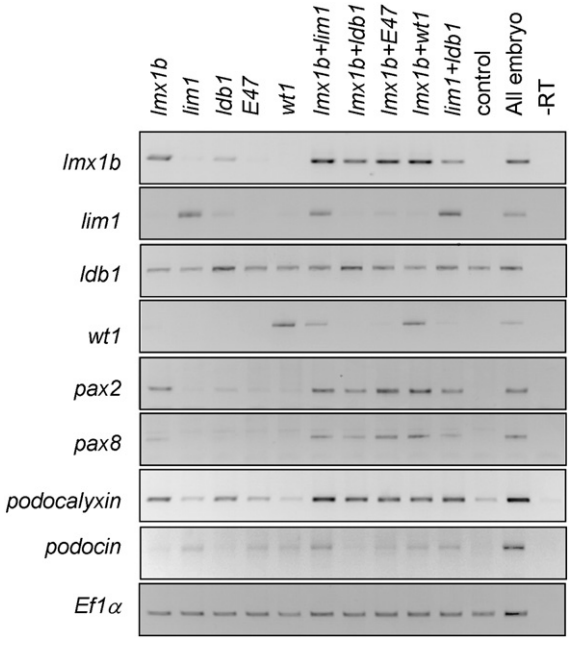

Fig. 8. Over-expression of *lmx1b* and its potential binding partners induces expression of kidney markers in animal cap assay. One-cell embryos were injected with mRNA encoding *lmx1b* and its potential binding partners either alone or in combination. Animal caps were removed at stage 8/9 and cultured until control embryos reached stage 26. Expression of kidney markers was assessed by RT-PCR. *Wt1* expression is up-regulated by co-injection of *lmx1b* and *lim1*. Expression of *pax-2* and *pax-8* is up-regulated by co-injection of *lmx1b* alone and in combination with its potential binding partners. Expression of *podocalyxin* is also up-regulated by co-injection of *lmx1b* and its potential binding partners. A weak up-regulation of *podocin* is also induced by co-injection of *lmx1b* and *lim1*. Over-expression of the injected exogenous mRNAs can still be detected in most samples at the time of harvesting. Over-expression of *E47* was not demonstrated since *in vitro* transcribed human *E47* mRNA, not detected by the primers used to amplify *Xenopus E47*, was used for the injection. *EF1a* was used as a loading control.

might be regulated by other pathways. These caps also expressed neural markers *pax6 hoxb9* and *otx2*, but the presence of podocyte markers unambiguously identifies kidney tissue in addition to neural tissue (data not shown).

Discussion

lmx1b is expressed in the right place and the right time to affect pronephric glomus development

There is currently little molecular knowledge of either the initial inducing molecules or even the patterning molecular networks which define the functional glomus. We have previously described the expression pattern and growth factor inducibility of the *lmx1b* gene (Haldin et al., 2003). Here we extend our published results of the gene expression pattern of *lmx1b* and describe the distribution of its transcripts in dissected pronephric primordia which indicate its expression when pronephric primordia are first specified and later primarily in the pronephric glomus (Brennan et al., 1998, 1999). Thus it is expressed at the right time and in the right place to play a developmental role in glomus development (Lamb et al., 1993).

lmx1b has a pivotal role in pronephric glomus development

We present new functional data which demonstrates clearly that *lmx1b* has a pivotal role in the development of the pronephric glomus. Morpholino oligonucleotide knock-down with two independent morpholinos affects the size of both the glomus and proximal tubule domains of the pronephros in an identical manner. Both morpholino phenotypes can be rescued with a mutant mRNA showing that the effect is specific to the gene tested. Knock-outs of *Lmx1b* have been achieved by both a non-conditional and a conditional strategy and analyses of the glomerular phenotype have been carried out in the metanephros of the newborn mice (Chen et al., 1998; Suleiman et al., 2007). These analyses clearly demonstrate that *Lmx1b* has a role in the differentiation of the podocyte foot processes which, together with the basement membrane, provide the size filtration barrier. Our data, which are the first to address the role of *lmx1b* in pronephric development, suggest that this gene has a previously unidentified role in the earlier differentiation of the pronephric glomus.

Previous studies have identified developmental roles for WT1 in glomus development and podocyte determination (Wallingford et al., 1998; Kreidberg et al., 1993; Kreidberg, 2003). Gene expression analysis in *Xenopus* shows that the Wilms tumor suppressor, *wt1*, is expressed from stage 20 in a ridge on the dorsal side of the tubule anlagen defining the presumptive pronephric glomus. At stage 35/36, both the glomus and the cells lining the coelom express this gene and by stage 38 the heart is also positive (Carroll et al., 1999). Over-expression of *wt1* disrupts glomus development and inhibits pronephric tubule formation. It has been hypothesised that *wt1* may function to reserve the fate of pronephric competent cells for the formation of the glomus, possibly by negative interactions with *pax2*, *lim1* and *Wnt4*, thus excluding their expression from the presumptive glomus (Wallingford et al., 1998). This is supported by the zebrafish model in which the *no isthmus* mutant, which lacks functional *pax2.1*, shows abnormally expanded podocyte marker gene expression suggesting that *pax2.1* negatively regulates *wt1* (Majumdar et al., 2000). This mutual repression sets up a boundary which defines glomerular and tubular epithelial territories. In mammals, *WT1* also acts as a positive regulator of the glomus markers *nephrin*, *podocalyxin* and *amphiregulin* and basement membrane components *Col4a3* and *Col4a4* (Palmer et al., 2001; Quaggin, 2002; Guo et al., 2004).

We attempted to identify podocyte structure in our experiments using antibodies to desmin previously shown to immunoreact with adult *Xenopus* kidney podocytes and laminin to identify kidney structures (Yaoita et al., 1999). These failed to interact with embryonic preparations, thus precluding a direct analysis of podocyte structure in these studies. Furthermore, embryos previously subjected to *in situ* analysis do not provide histological samples of high quality due to the essential proteinase K steps, preventing histological analysis of phenotypically affected embryos. However, due to the observed effects on two podocyte molecular markers *wt1* and *nephrin*, as in higher vertebrates we expect the podocyte structure and organisation to be disrupted.

The importance of the balance in expression levels of lmx1b and other LIM domain binding partners in regulating glomus and tubule development

It is well known that LIM domain proteins need additional cofactors to both exert their transcriptional effects and also to determine their tissue specificity (Dawid et al., 1998). Recent studies have identified that Lmx1b binds to two such cofactors, Ldb1 and the helix–loop helix protein, E47 (German et al., 1992; Dreyer et al., 2000; Marini et al., 2003). Lim1 has also been shown to bind to Ldb1 both *in vitro* and *in vivo* (Agulnick et al., 1996). Our data provide new insight into the role of expression levels of lmx1b and its binding partners in regulating glomus versus tubule development.

Lmx1b and Lim1 have been shown to interact in mesonephros and metanephros formation and patterning, but there is no information as to their roles in the formation of the initial kidney form, the pronephros, due to the inaccessibility of the pronephros or the embryonic lethality of the gene knock-outs.

Suleiman et al. (2007), have recently reported the phenotypes of a series of podocyte-specific conditional knock-out mouse lines for *Lmx1b* and its potential binding partners. The *Lmx1b* line mimics more closely the phenotype observed in NPS than the conventional *Lmx1b* knock-out mouse; the pups do not die at birth and do not show down-regulation of *Col4a3*, *Col4a4* and *Nphs2* genes. Furthermore, these authors generated a podocyte specific *Ldb1* knock-out line which has a similar phenotype suggesting that Ldb1 is the binding partner for Lmx1b in the glomerulus. Our data supports this conclusion, but in the earlier kidney form, the pronephros.

We have shown in the *Xenopus* system that both during development of the pronephric anlagen and all the components of dissected pronephric material that *ldb1* is expressed throughout the period that the kidney forms, and that its potential binding partners *lim1* and *lmx1b* are temporally co-expressed. This information is not available in the developing mouse kidney where the exact distribution of Ldb1 is uncertain (Suleiman et al., 2007). Our experiments indicate that *lmx1b* is expressed in advance of *nephrin* and *wt1* and also when ectopically expressed can increase *wt1* and *nephrin* expression *in vivo*. Furthermore, morpholino knock-down of *lmx1b* results in reduction of glomus size. Animal cap studies also indicate that over-expression of *lmx1b* and *lim1* can up-regulate *wt1* whereas *wt1* over-expression fails to up-regulate *lmx1b*. These data suggest that *lmx1b* acts upstream of *wt1* in early glomus development. Up-regulation of *wt1* could then potentially inhibit expression of *pax2/8*, *lim1* and *wnt4* allowing the separation of lateral pronephric mesoderm to give tubules under the influence of lim1 and ldb1 and splanchnic pronephric mesoderm to develop into glomus.

Minimal effects were seen on co-injection of *E47* and *lmx1b* suggesting that *E47* has no major role in kidney development, this is consistent with mammalian knock-out studies where either conventional or conditional knock-outs fail to show a kidney phenotype, even though E47 is able to interact with *Lmx1b* (Zhuang et al., 1994; Johnson et al., 1997; Jurata and Gill, 1997; Suleiman et al., 2007).

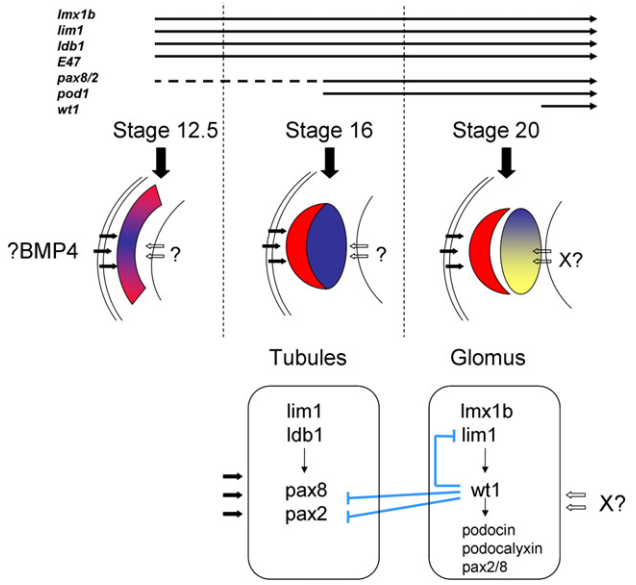

Fig. 9. Working model for glomus and tubule allocation in *Xenopus*. *lmx1b*, *lim1*, *ldb1* and *E47* are expressed from late gastrula until medio/lateral separation of the glomus occurs at stage 20 in the pronephric primordial. *Pax2/8* are up-regulated at about stage 15 and become restricted to the tubule primordium by stage 20. Podocyte-specific genes *pod1* and *wt1* are up-regulated at stage 15 and 20 respectively. The spatial changes in the unpatterned pronephric primordium are shown to reflect initially the separation of the proximal tubule from the more distal tubule elements and then their medio/lateral separation from the glomus. Gene interactions in the tubule separation from the glomus are shown together with putative interactions from epidermal and endodermal components. *lim1* and *ldb1* interact and up-regulate tubule specific genes, *pax2/8*. *lmx1b* and *lim1* interact to up-regulate *wt1*, a marker of podocytes, which in turn down regulates *lim1*, *pax2/82* to define the glomus domain. Wt1 up-regulates other markers of podocyte differentiation. Gene interactions indicated in blue are from previously published data (see text).

The genetic hierarchy involved in glomus development — a model

The expression profiles of transcription factors in early pronephric structures define to an extent, the roles they may have in the transcriptional network controlling differentiation. Several large scale screens have been carried out to identify genes involved in glomerular development (Cui et al., 2005; Takemoto et al., 2006) however little has been done in the pronephric glomus. Our animal cap experiments investigate the ability of combinations of these transcription factors to up-regulate pronephric downstream target genes. Our experiments indicate that co-injection of *lim1* and *ldb1* is able to switch on the expression of *pax8* a marker of proximal and more distal tubule differentiation in the early pronephric anlagen. Over-expression of *pax8* and *lim1* has been shown to be able to induce the development of ectopic tubules indicating the importance of these genes in the development of tubules (Carroll and Vize, 1999). In addition, our data show that co-injection of *lmx1b* and *lim1* can up-regulate the expression of *wt1*, a transcription factor which marks medio-lateral separation of the glomus from the tubule anlagen. In mammals, several directly defined downstream targets of WT1 have been defined in glomerular development and podocyte differentiation (Palmer et al., 2001; Guo et al., 2004). Likewise some downstream targets of Lmx1b have been identified to include podocyte developmental genes (Rohr et al., 2002). We propose that lmx1b in combination with lim1 or ldb1 performs a fundamental role controlling the differentiation of the glomus away from the tubule components, tubule differentiation on the other hand, being driven by the other LIM protein lim1, in combination with ldb1. While it is unknown what signals are responsible for the medio-lateral separation of the elements of the pronephros, we can speculate that epidermal signals either restrict the lmx1b:ldb1 interaction and thus *wt1* expression, to the medio-lateral domain, or stabilise/up-regulate the lim1:ldb1 interaction leading to *pax8* expression (Vize, 2003a; Urban et al., 2006). There is evidence from the elegant work of Urban et al, that hedgehog signals over-expressed in the ectoderm overlying the pronephric anlagen inhibit the segregation of the pronephric anlagen from the intermediate mesoderm without affecting the pronephric glomus (Urban et al., 2006). This work establishes the feasibility of such a patterning event occurring *in vivo*. Additionally, it is possible that medial signals are received in the splanchnic mesoderm from the adjacent endoderm to help pattern this region, although the postulated nature of these signals is unknown (Fig. 9).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2008.07.012.

References

Agulnick, A.D., Taira, M., Breen, J.J., Tanaka, T., Dawid, I.B., Westphal, H., 1996. Interactions of the LIM-domain-binding factor Ldb1 with LIM homeodomain proteins. Nature 384, 270–272.

Barnett, M.W., Old, R.W., Jones, E.A., 1998. Neural induction and patterning by fibroblast growth factor, notochord and somite tissue in *Xenopus*. Dev. Growth Differ. 40 (1), 47–57.

Bassez, T., Paris, J., Omilli, F., Dorel, C., Osborne, H.B., 1990. Post-transcriptional regulation of ornithine decarboxylase in *Xenopus laevis* oocytes. Development 110 (3), 955–962.

Boute, N., Gribouval, O., Boute, N., Sich, M., Benessy, F., et al., 2000. NPHS2, encoding the glomerular protein podocin, is mutated in autosomal recessive steroid resistant nephrotic syndrome. Nat. Genet. 24, 349–354.

Brändli, A.W., 1999. Towards a molecular anatomy of the *Xenopus* pronephric kidney. Int. J. Dev. Biol. 43, 381–395.

Brennan, H.C., Nijjar, S., Jones, E.A., 1998. The specification of the pronephric tubules and duct in *Xenopus laevis*. Mech. Dev. 75, 127–137.

Brennan, H.C., Nijjar, S., Jones, E.A., 1999. The specification and growth factor inducibility of the pronephric glomus in *Xenopus laevis*. Development 126, 5847–5856.

Carroll, T.J., Vize, P.D., 1996. Wilms tumor suppressor gene is involved in the development of disparate kidney forms: evidence from expression in the *Xenopus* pronephros. Dev. Dyn. 206, 131–138.

Carroll, T.J., Vize, P.D., 1999. Synergism between Pax-8 and Lim-1 in embryonic development. Dev. Biol. 214, 46–59.

Carroll, T.J., Wallingford, J., Seufert, D., Vize, P.D., 1999. Dynamic patterns of gene expression in the developing pronephros of *Xenopus laevis*. Dev Genet. 24, 199–207.

Chan, T.C., Takahashi, S., Asashima, M., 2000. A role for Xlim-1 in pronephros development in *Xenopus laevis*. Dev. Biol. 15, 256–269.

Chen, H., Lun, Y., Ovchinnikov, D., Kokubo, H., Oberg, K.C., Pepicelli, C.V., Gan, L., Lee, B., Johnson, R.L., 1998. Limb and kidney defects in Lmx1b mutant mice suggest an involvement of LMX1B in human nail patella syndrome. Nat.Genet. 19, 51–55.

Clough, M.V., Hamlington, J.D., McIntosh, I., 1999. Restricted distribution of loss-of-function mutations within the LMX1B genes of nail-patella syndrome patients. Hum. Mutat. 14 (6), 459–465.

Cui, S., Li, C., Ema, M., Weinstein, J., Quaggin, S.E., 2005. Rapid isolation of glomeruli coupled with gene expression profiling identifies downstream targets in Pod1 knockout mice. J. Am. Soc. Nephrol. 16, 3247–3255.

Dawid, I.B., Breen, J.J., Toyama, R., 1998. LIM domains: multiple roles as adapters and functional modifiers in protein interactions. Trends Genet. 14, 156–162.

Dreyer, S.D., Zhou, G., Baldini, A., Winterpacht, A., Zabel, B., Cole, W., Johnson, R.L., Lee, B., 1998. Mutations in LMX1B cause abnormal skeletal patterning and renal dysplasia in nail patella syndrome. Nat. Genet. 19, 51–55.

Dreyer, S.D., Morello, R., German, M.S., Zabel, B., Winterpacht, A., Lunstrum, G.P., Horton, W.A., Oberg, K.C., Lee, B., 2000. LMX1B transactivation and expression in nail-patella syndrome. Hum. Mol. Genet. 9, 1067–1074.

Drummond, I.A., Majumdar, A., 2003. The pronephric glomus and vasculature, The Kidney: From Normal Development to Congenital Disease, 1st ed. Elsevier Science, Academic Press, London, pp. 61–73.

Dunston, J.A., Hamlington, J.D., Zaveri, J., Sweeney, E., Sibbring, J., Tran, C., Malbroux,

M., O'Neill, J.P., Mountford, R., McIntosh, I., 2004. The human LMX1B gene: transcription unit, promoter, and pathogenic mutations. Genomics 84, 565–576.
Eid, S.R., Brändli, A.W., 2001. *Xenopus* Na,K-ATPase: primary sequence of the beta2 subunit and in situ localization of alpha1, beta1, and gamma expression during pronephric kidney development. Differentiation 68 (2–3), 115–125.
Field, H.H., 1891. The development of the pronephros and segmental duct in Amphibia. Bull. Mus. Comp. Zool. (Harvard College) Vol. XXI (5).
German, M.S., Wang, J., Chadwick, R.B., Rutter, W.J., 1992. Synergistic activation of the insulin gene by a LIM-homeodomain protein and a basic helix-loop-helix protein: building a functional insulin minienhancer complex. Genes Dev. 6, 2165–2176.
Gerth, V.E., Zhou, X., Vize, P.D., 2005. Nephrin expression and three-dimensional morphogenesis of the *Xenopus* pronephric glomus. Dev Dyn. 233 (3), 1131–1139.
Guo, G., Morrison, D.J., Licht, J.D., Quaggin, S.E., 2004. WT1 activates a glomerular-specific enhancer identified from the human nephrin gene. J. Am. Soc. Nephrol. 15, 2851–2856.
Haldin, C.E., Nijjar, S., Massé, K., Barnett, M.W., Jones, E.A., 2003. Isolation and growth factor inducibility of the *Xenopus laevis* Lmx1b gene. Int. J. Dev. Biol. 47, 253–262.
Harland, R.M., 1991. *In situ* hybridisation — an improved whole mount method for *Xenopus* embryos. Methods Cell Biol. 36, 685–695.
Heller, N., Brändli, A.W., 1999. *Xenopus Pax-2/5/8* orthologues: novel insights into Pax gene evolution and identification of Pax-8 as the earliest marker for otic and pronephric cell lineages. Dev. Genet. 24 (3–4), 208–219.
Huang, S., Johnson, K.E., Wang, H.Z., 1998. Blastomeres show differential fate changes in 8-cell *Xenopus laevis* embryos that are rotated 90 degrees before first cleavage. Dev Growth Differ. 40, 189–198.
Johnson, J.D., Zhang, W., Rudnick, A., Rutter, W.J., German, M.S., 1997. Transcriptional synergy between LIM-homeodomain proteins and basic helix–oop–helix proteins: the LIM2 domain determines specificity. Mol. Cell. Biol. 17, 3488–3496.
Jones, E.A., 2003. Molecular control of pronephric development: and overview, The Kidney: From Normal Development to Congenital Disease, 1st ed. Elsevier Science, Academic Press, London, pp. 93–118.
Jurata, L.W., Gill, G.N., 1997. Functional analysis of the Nuclear LIM Domain interactor NLI. Mol. Cell. Biol. 17, 5688–5698.
Knoers, N.V., Bongers, E.M., van Beersum, S.E., Lommen, E.J., van Bokhoven, H., Hol, F.A., 2000. Nail-patella syndrome: identification of mutations in the LMX1B gene in Dutch families. J. Am. Soc. Nephrol. 11 (9), 1762–1766.
Kreidberg, J.A., 2003. Podocyte differentiation and glomerulogenesis. J. Am. Soc. Nephrol. 14, 806–814.
Kreidberg, J.A., Sariola, H., Loring, J.M., Maeda, M., Pelletier, J., Housman, D., Jaenisch, R., 1993. WT-1 is required for early kidney development. Cell 2, 679–691.
Kyuno, J., Fukui, A., Michiue, T., Asashima, M., 2003. Identification and characterization of *Xenopus* NDRG1. Biochem. Biophys. Res. Commun. 309 (1), 52–57.
Lamb, T.M., Knecht, A.K., Smith, W.C., Stachel, S.E., Economides, A.N., Stahl, N., Yancopolous, G.D., Harland, R.M., 1993. Neural induction by the secreted polypeptide noggin. Science 262, 713–718.
Majumdar, A., Lun, K., Brand, M., Drummond, I.A., 2000. Zebrafish no isthmus reveals a role for pax2.1 in tubule differentiation and patterning events in the pronephric primordia. Development 127, 2089–2098.
Marini, M., Bongers, E.M., Cusano, R., Di Duca, M., Seri, M., Knoers, N.V., Ravazzolo, R., 2003. Confirmation of CLIM2/LMX1B interaction by yeast two-hybrid screening and analysis of its involvement in nail-patella syndrome. Int. J. Mol. Med. 12, 79–82.
McIntosh, I., Dreyer, S.D., Clough, M.V., Dunston, J.A., Eyaid, W., Roig, C.M., Montgomery, T., Ala-Mello, S., Kaitila, I., Winterpacht, A., Zabel, B., Frydman, M., Cole, W.G., Francomano, C.A., Lee, B., 1998. Mutation analysis of LMX1B gene in nail-patella syndrome patients. Am. J. Hum. Genet. 63 (6), 1651–1658.
Millá, E., Hernan, I., Gamundi, M.J., Martínez-Gimeno, M., Carballo, M., 2007. Novel LMX1B mutation in familial nail-patella syndrome with variable expression of open angle glaucoma. Mol. Vis. 13, 639–648.
Miner, J.H., Morello, R., Andrews, K.L., Li, C., Antignac, C., Shaw, A.S., Lee, B., 2002. Transcriptional induction of slit diaphragm genes by Lmx1b is required in podocyte differentiation. J. Clin. Invest. 109, 1065–1072.
Mohun, T.J., Taylor, M.V., Garrett, N., Gurdon, J.B., 1989. The CArG promoter sequence is necessary for muscle-specific transcription of the cardiac actin gene in *Xenopus* embryos. EMBO J. 8, 1153–1161.
Moody, S.A., Kline, M.J., 1990. Segregation of fate during cleavage of frog (*Xenopus laevis*) blastomeres. Anat. Embryol. (Berl) 182, 347–362.
Nieuwkoop, P.D., Faber, J., 1994. Normal table of *Xenopus laevis* (Daudin). Garland, New York.
P - almer, R.E., Kotsianti, A., Cadman, B., Boyd, T., Gerald, W., Haber, D.A., 2001. WT1 regulates the expression of the major glomerular podocyte membrane protein Podocalyxin. Curr. Biol. 11, 1805–1809.
Quaggin, S.E., 2002. Transcriptional regulation of podocyte specification and differentiation. Micros. Res. Tech. 57, 208–211.
Reggiani, L., Raciti, D., Airik, R., Kispert, A., Brändli, A.W., 2007. The prepattern transcription factor Irx3 directs nephron segment identity. Genes Dev. 21 (18), 2358–2370.
Riddle, R.D., Ensini, M., Nelson, C., Tsuchida, T., Jessell, T.M., Tabin, C., 1995. Induction of the LIM homeobox gene Lmx1 by WNT7a establishes dorsoventral pattern in the vertebrate limb. Cell 83, 631–640.
Rohr, C., Prestel, J., Heidet, L., Hosser, H., Kriz, W., Johnson, R.L., Antignac, C., Witzgall, R., 2002. The LIM-homeodomain transcription factor Lmx1b plays a crucial role in podocytes. J. Clin. Invest. 109, 1073–1082.
Sato, A., Asashima, M., Yokota, T., Nishinakamura, R., 2000. Cloning and expression pattern of a *Xenopus* pronephros-specific gene, XSMP-30. Mech. Dev. 92 (2), 273–275.
Saxén, L., 1987. Organogenesis of the Kidney, 1st ed. Cambridge University Press.
Seville, R.A., Nijjar, S., Barnett, M.W., Massé, K., Jones, E.A., 2002. Annexin IV (*Xanx-4*) has a functional role in the formation of pronephric tubules. Development 129, 1693–1704.
Shawlot, W., Behringer, R.R., 1995. Requirement for Lim1 in head-organizer function. Nature 374, 425–430.
Simrick, S., Massé, K., Jones, E.A., 2005. Developmental expression of *Pod 1* in *Xenopus laevis*. Int. J. Dev. Biol. 49, 59–63.
Suleiman, H., Heudobler, D., Raschta, A.-S., Zhao, Y., Zhao, Q., Hertting, I., Vitzthum, H., Moeller, M.J., Holzman, L.B., Rachel, R., Johnson, R., Westphal, H., Rascle, A., Witzgall, R., 2007. The podocyte-specific inactivation of Lmx1b, Ldb1 and E2a yields new insight into a transcriptional network in podocytes. Dev. Biol. 304, 701–712.
Sweeney, E., Fryer, A., Mountford, R., Green, A., McIntosh, I., 2003. Nail patella syndrome: a review of the phenotype aided by developmental biology. J. Med. Genet. 40, 153–162.
Taira, M., Jamrich, M., Good, P.J., Dawid, I.B., 1992. The LIM domain-containing homeo box gene *Xlim-1* is expressed specifically in the organiser region of *Xenopus* gastrula embryos. Genes Dev. 6, 356–366.
Taira, M., Otani, H., Jamrich, M., Dawid, I.B., 1994. Expression of the LIM class homeobox gene *Xlim-1* in pronephros and CNS cell lineages of *Xenopus* embryos is affected by retinoic acid and exogastrulation. Development 120, 1525–1536.
Takemoto, M., He, L., Norlin, J., Patrakka, J., Xiao, Z., Petrova, T., Bondjers, C., Asp, J., Wallgard, E., Sun, Y., Samuelsson, T., Mostad, P., Lundin, S., Miura, N., Sado, Y., Alitalo, K., Quaggin, S.E., Tryggvason, K., Betsholtz, C., 2006. Large-scale identification of genes implicated in kidney glomerulus development and function. EMBO J. 25, 1160–1174.
Urban, A.E., Zhou, X., Ungos, J.M., Raible, D.W., Altmann, C.R., Vize, P.D., 2006. FGF is essential for both condensation and mesenchymal-epithelial transition stages of pronephric kidney tubule development. Dev. Biol. 297, 103–117.
Vize, P.D., 2003a. Induction, Development and Physiology of the pronephric tubules, The kidney: From Normal Development to Congenital Disease, 1st ed. Elsevier Science, Academic Press, London, pp. 19–50.
Vize, P.D., 2003b. The chloride conductance channel ClC-K is a specific marker for the *Xenopus* pronephric distal tubule and duct. Gene Expr. Patterns 3 (3), 347–350.
Vize, P.D., Jones, E.A., Pfister, R., 1995. Development of the *Xenopus* pronephric system. Dev. Biol. 171, 531–554.
Vize, P.D., Seufert, D.W., Carroll, T.J., Wallingford, J.B., 1997. Model systems for the study of kidney development: use of the pronephros in the analysis of organ induction and patterning. Dev. Biol. 188, 189–204.
Vollrath, D., Jaramillo-Babb, V.L., Clough, M.V., McIntosh, I., Scott, K.M., Lichter, P.R., Richards, J.E., 1998. Loss-of-function mutations in the LIM-homeodomain gene, LMX1B, in nail-patella syndrome. Hum. Mol. Genet. 7 (7), 1091–1098 Erratum in: Hum Mol Genet 1998 Aug;7(8):1333.
Wallingford, J.B., Carroll, T.J., Vize, P.D., 1998. Precocious expression of the Wilms' tumor gene *xWT1* inhibits embryonic kidney development in *Xenopus laevis*. Dev. Biol. 202, 103–112.
Witta, S.E., Sato, S.M., 1997. XIPOU 2 is a potential regulator of Spemann's Organizer. Development 124, 1179–1189.
Yaoita, E., Franke, W.W., Yamamoto, T., Kawasaki, K., Kihara, I., 1999. Identification of renal podocytes in multiple species: higher vertebrates are vimentin positive/lower vertebrates are desmin positive. Histochem. Cell Biol. 111 (2), 107–115.
Zhuang, Y., Soriano, P., Weintraub, H., 1994. The helix–loop helix gene EA is required for B cell formation. Cell 79, 875–884.