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The nephrogenic potential of the transcription factors *osr1*, *osr2*, *hnf1b*, *lhx1* and *pax8* assessed in *Xenopus* animal caps

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Abstract

Background: The three distinct types of kidneys, pronephros, mesonephros and metanephros, develop consecutively in vertebrates. The earliest form of embryonic kidney, the pronephros, is derived from intermediate mesoderm and the first expressed genes localized in the pronephros anlage are the transcription factors *osr1*, *osr2*, *hnf1b*, *lhx1* and *pax8*, here referred to as the early nephrogenic transcription factors. However, the pathway inducing nephrogenesis and the network of these factors are poorly understood. Treatment of the undifferentiated animal pole explant (animal cap) of *Xenopus* with activin A and retinoic acid induces pronephros formation providing a powerful tool to analyze key molecular events in nephrogenesis.

Results: We have investigated the expression kinetics of the early nephrogenic transcription factors in activin A and retinoic acid treated animal caps and their potential to induce pronephric differentiation. In treated animal caps, expression of *osr1*, *osr2*, *hnf1b* and *lhx1* are induced early, whereas *pax8* expression occurs later implying an indirect activation. Activin A alone is able to induce *osr2* and *lhx1* after three hours treatment in animal caps while retinoic acid fails to induce any of these nephrogenic transcription factors. The early expression of the five transcription factors and their interference with pronephros development when overexpressed in embryos suggest that these factors potentially induce nephrogenesis upon expression in animal caps. But no pronephros development is achieved by either overexpression of *OSR1*, by *HNF1B* injection with activin A treatment, or the combined application of *LHX1* and *PAX8*, although they influenced the expression of several early nephrogenic transcription factors in some cases. In an additional approach we could show that *HNF1B* induces several genes important in nephrogenesis and regulates *lhx1* expression by an *HNF1* binding site in the *lhx1* promoter.

Conclusions: The early nephrogenic transcription factors play an important role in nephrogenesis, but have no pronephros induction potential upon overexpression in animal caps. They activate transcriptional cascades that partially reflect the gene activation initiated by activin A and retinoic acid. Significantly, *HNF1B* activates the *lhx1* promoter directly, thus extending the known activin A regulation of the *lhx1* gene via an activin A responsive element.

Background

During vertebrate development three kidney types of increasing complexity (pronephros, mesonephros and metanephros) form successively from the intermediate mesoderm, located between the paraxial mesoderm (developing somites) and the lateral plate [1]. The pronephros is the simplest, functional form of kidney in larval stages of fish and amphibians and consists of three

major components: glomus, tubules and duct. In adults the pronephros is replaced by the mesonephros. In mammals the pronephros is not functional, but required for mesonephros formation that is replaced by the metanephros, the kidney of the adult [2].

All components of the pronephros arise from intermediate mesoderm, but the signals that direct patterning of the presumptive pronephric mesoderm towards pronephric lineages are unknown. Experiments showed that the anterior somites are crucial for pronephros development and provide an essential first signal. If the anterior

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somites are removed [3] or separated from the presumptive pronephros [4], pronephroi do not form. Anterior somites can also induce pronephric tubules in unspecified intermediate mesoderm [3]. Although the exact timing and nature of the signal provided by the anterior somites are yet unknown, *wnt11b* expressed throughout the anterior somites has recently been shown as a crucial signal [5].

Xenopus is a very attractive model organism to analyze key molecular events in nephrogenesis, because most genes essential for pronephros development in *Xenopus* embryos are also crucial for the formation of the more complex mammalian kidneys [6-9]. A classical method to identify important molecules in *Xenopus* development is the injection of mRNAs or morpholino oligonucleotides into the fertilized egg or into blastomeres of early cleaving stages [10,11]. Thus, several pronephric regulators have been functionally identified [7,8,12]. An additional experimental tool to study early events of nephrogenesis involves explanting the animal pole of the blastula. These explanted animal caps have pluripotency and differentiate into various tissues upon exposure to inducing substances [13,14]. Importantly, animal caps treated with activin A and retinoic acid differentiate into pronephros [15] and in this *in vitro* system genes are induced with similar kinetics as *in vivo* [16-18].

In *Xenopus* the first genes expressed in the pronephros anlage are the transcription factors *osr1* and *osr2*, members of the odd-skipped family of proteins [19], *hnf1b*, a member of the homeobox factors [20], *lhx1* (*lim1*), a *lim* homeobox factor [21] as well as *pax8*, a member of the paired box domain family [22]. We refer to these five transcription factors as the early nephrogenic transcription factors, as they are all expressed in the pronephros anlage prior to cellular differentiation and their misexpression affects pronephros development. Inhibition of *osr1* or *osr2* by morpholinos in *Xenopus* embryos interferes with kidney formation and embryonic overexpression of either of these factors induces ectopic kidney tissue and enlarged pronephros [19]. Overexpression of *hnf1b* inhibits pronephros formation [23] and this effect is also seen by using the human HNF1B [24] implying that the regulatory potential has been conserved during vertebrate evolution. In contrast, *lhx1* and *pax8* overexpression leads to an enlargement of the pronephros and, if both factors are coexpressed, this effect is increased and even induces ectopic pronephric tubules [25].

It should be noted that each of these five early nephrogenic transcription factors plays also a crucial role in the development of other organs. The prominent role of these nephrogenic transcription factors is partially also evident in mammalian systems. Whereas null

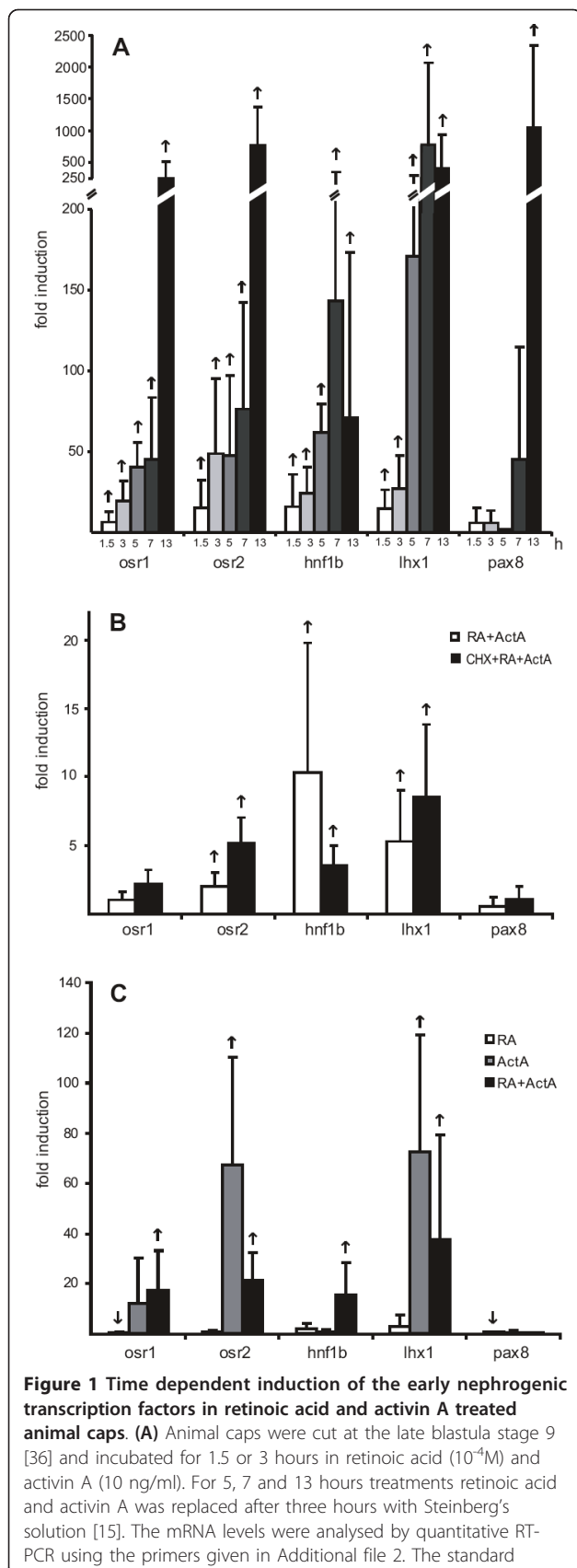
mutation of *Osr1* in mice exhibit agenesis of the kidney [26], *Osr2* knock-out has no kidney phenotype [27], although *Osr2* transcripts are expressed in the developing kidney [28]. The kidney-restricted knockout of *Hnf1b* leads to polycystic kidney disease [29] and the *Lhx1* null mutant even lacks any kidney [30]. In contrast, *Pax8* deficient mice exhibit thyroid gland deficiency, but have no pronephric phenotype [31]. Nevertheless, *Pax8* plays an essential role in kidney development, as impaired metanephros formation observed in mice deficient for *Pax2* [32] is dramatically increased by a lack of any nephric cell lineage, if these embryos lack additionally *Pax8* [33].

To further explore the role of these five nephrogenic transcription factors we have now analyzed the kinetics of their induction in animal caps differentiated into pronephric tissue by activin A and retinoic acid. We then have overexpressed these transcription factors in animal caps and analyzed their potential to induce each other and to stimulate pronephric differentiation in these explants. To allow discrimination between injected mRNAs and endogenous mRNAs we used the human mRNAs that are functionally equivalent, but are not detectable with the *Xenopus* probes. We use capital letters for these human transcription factors to make a clear distinction. In addition we identified genes induced by HNF1B in these early embryonic cells.

Results

Induction of mRNAs encoding the early nephrogenic transcription factors *osr1*, *osr2*, *hnf1b*, *lhx1* and *pax8* in animal caps treated with retinoic acid and/or activin A

Since simultaneous treatment of *Xenopus* animal caps with 10 ng/ml of activin A and 10^{-4} M retinoic acid for three hours induces differentiation of pronephric tissues [15], we explored the time dependent induction of the early nephrogenic transcription factors *osr1*, *osr2*, *hnf1b*, *lhx1* and *pax8* in these embryonic explants. Thus, we measured by quantitative RT-PCR the induction of the corresponding mRNAs in animal caps treated with retinoic acid and activin A (Figure 1A). Based on the low sample pools ($N = 4$) and because we do not know whether the values obtained in various experiments with animal caps represent a normal distribution, the use of a significance test is not appropriate. Therefore, we defined genes as induced or repressed, when all the four measured values for a given sample pool represented the same trend. Using these criteria all five transcripts were induced, albeit with different kinetics. Whereas *osr1*, *osr2*, *hnf1b* and *lhx1* mRNAs were induced within 1.5 hours, *pax8* mRNA was only increased after thirteen hours. For *osr1* and *osr2* the induction increased up to thirteen hour treatment, whereas *hnf1b* and *lhx1* seemed to level off at seven hour treatment. By inhibition of protein synthesis with cycloheximide, the



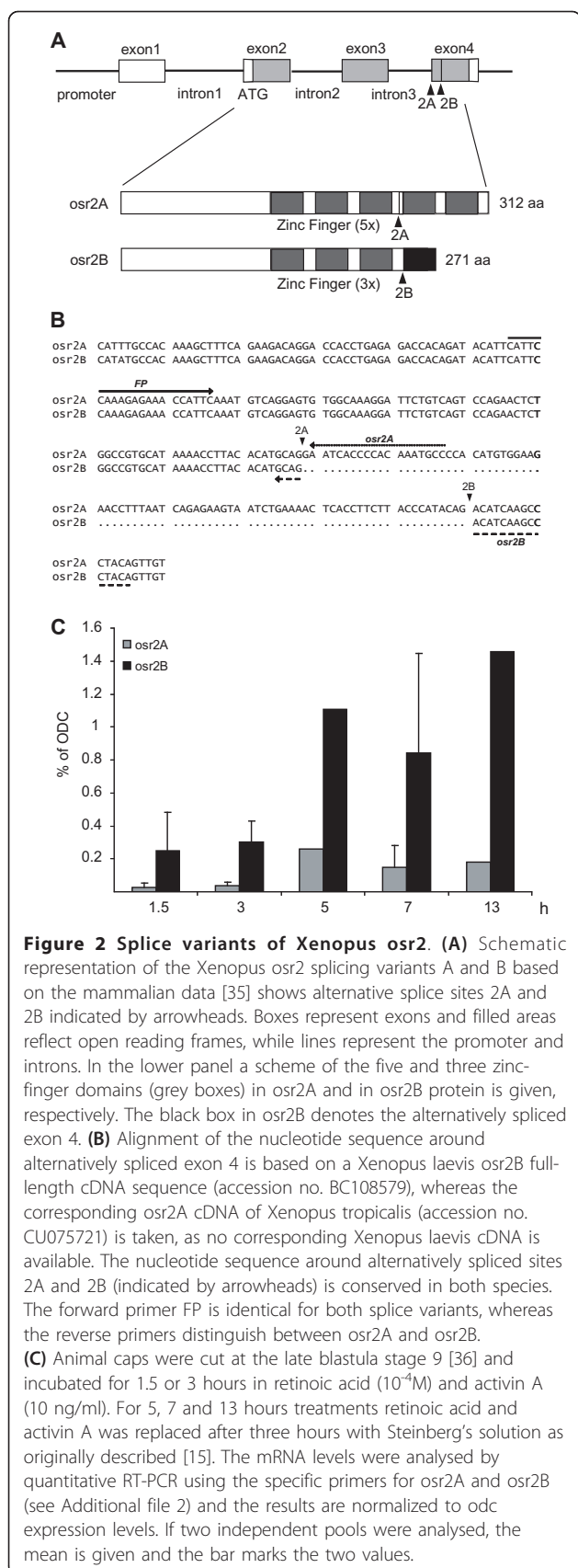
deviations from four independent animal cap pools (N = 30) are given and alterations are indicated by an upward arrow, if all four induction values were higher than one for a given probe. To detect *osr2* transcripts primers targeting *osr2B*, the splice variant predominantly expressed in *Xenopus laevis* (compare Figure 2B) were used for two animal cap pools analyzed after 3, 5 and 7 hours and three after 13 hours. In all other experiments primers targeting both splice variants were used. (B) Animal caps cut at the late blastula stage 9 were incubated with or without cycloheximide (CHX, 5 μ g/ml) for 30 min and then stimulated for 1.5 hours in retinoic acid (10^{-4} M) and activin A (10 ng/ml). RNA was quantified from six pools (N = 30) and upward arrows indicate induction for all six experiments. (C) Animal caps were cultured for three hours in retinoic acid (RA, 10^{-4} M), activin A (ActA, 10 ng/ml) or both inducers together (RA+ActA) and then analysed by quantitative RT-PCR. The standard deviations from five (RA) or four (ActA or RA+ActA) independent animal cap pools (N = 30) are given and reproducible induction or reduction is indicated by an upward or downward arrow, if increased or decreased in all samples, respectively.

induction of *osr2*, *hnf1b* and *lhx1* was not abolished implying direct gene activation (Figure 1B). The observation that *osr1* was not induced in this experiment possibly reflects the 30 min culture with or without cycloheximide prior to retinoic acid and activin A treatment, since delayed activin A treatment of animal caps reduces the induction potential [34].

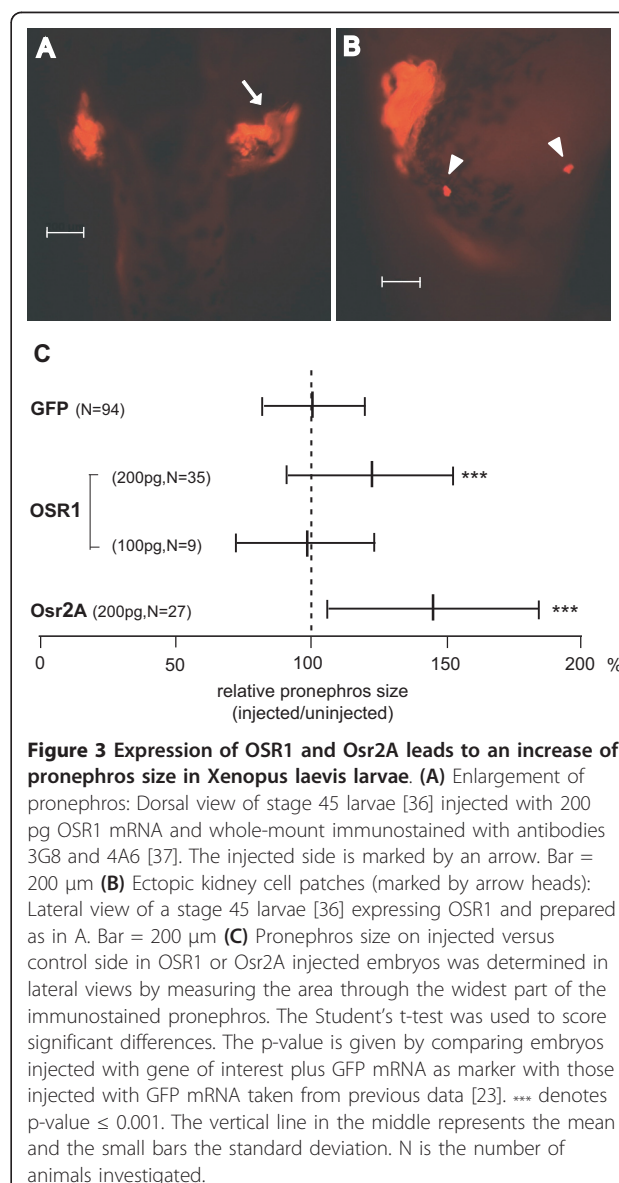
To clarify which nephrogenic transcription factor transcripts are induced by retinoic acid or activin A alone, we analyzed animal caps treated with either retinoic acid or activin A for three hours (Figure 1C). Treatment with retinoic acid failed to induce the nephrogenic transcription factors, but rather decreased the level of *osr1* and *pax8* transcripts by 2-fold and 1.6-fold, respectively. In contrast, activin A treatment induced *osr2* as well as *lhx1*, but *osr1* in two of four experiments only. Since *hnf1b* was not induced by activin A alone, we conclude that a synergistic effect of both inducers is needed to induce *hnf1b*. The lack of induction of *pax8* in animal caps treated with activin A or retinoic acid alone is consistent with the no-induction observed in animal caps treated with retinoic acid and activin A for three hours (also compare Figure 1A).

Overexpression of OSR1 and Osr2A leads to enlargement of pronephros and ectopic pronephric tissue

Since the murine *Osr2* gene is expressed in two splice variants referred to as *Osr2A* and *Osr2B* [35], we searched *Xenopus* cDNA sequences deposited in GeneBank for corresponding splice variants. Indeed, we identified transcripts encoding the A and B splice variants that encode a five and three finger zinc protein, respectively, comparable to the murine situation (Figure 2A and 2B). Using primers specific for the A and B splice variant we could show that *osr2B* predominates *osr2A* by a factor of about three throughout retinoic acid and activin A induction in animal caps (Figure 2C).



To examine the morphogenetic potential of the mammalian Osr1 and Osr2A in developing *Xenopus* embryos, we injected mRNA encoding the human OSR1 or the murine Osr2A proteins into one blastomere of the two-cell stage embryo using GFP mRNA as a marker to identify the injected side. About half of the OSR1 and more than the half of the Osr2A injected embryos showed gastrulation defects that were more severe with higher doses of mRNA. Injected embryos surviving to the free swimming tadpole stage 45 [36] were immunostained for pronephros development using a mixture of the monoclonal antibodies for the proximal tubules (3G8) and the distal tubules and pronephric duct (4A6) [37]. The majority of embryos injected with OSR1 showed an enlarged pronephros (Figure 3A), in some cases together with the formation of ectopic kidney



cell patches (Figure 3B). Comparing the size of the pronephros of the injected side with the one of the non-injected side we observed that the pronephros size was significantly enlarged by injecting 200 pg OSR1 but not with 100 pg (Figure 3C). Similarly, *Osr2A* overexpression (200 pg) leads to a significant increase of pronephros size as well, but we did not observe the formation of ectopic kidney tissue. In conclusion, OSR1 as well as *Osr2A* overexpression induces pronephros enlargement in *Xenopus* embryos as found previously for the corresponding *Xenopus* factors *osr1* and *osr2B* suggesting comparable function of both mammalian genes in pronephros development [19].

Overexpression of OSR1 alone or in combination with retinoic acid or activin A cannot support pronephros differentiation in animal caps

Since in our experiments expression of OSR1 had a lower lethality compared to *Osr2A* and induces ectopic

kidney tissues (Figure 3B), we investigated the potential of OSR1 to trigger pronephric differentiation in animal caps. Therefore, we injected mRNA encoding human OSR1 into the animal pole of two-cell stage embryos, explanted animal caps at late blastulae and cultured the explants for four days to monitor pronephros differentiation by immunostaining with the pronephros specific antibodies. In a control experiment without mRNA injection, but adding retinoic acid as well as activin A, pronephric differentiation was observed. Since the extent of differentiation was quite variable between individual caps, we scored the explants into three categories as illustrated in Figure 4A. Using the distribution into these categories it is clear that retinoic acid combined with activin A induced pronephric differentiation compared to untreated animal caps (Figure 4C), demonstrating kidney differentiation *in vitro* as previously reported [15]. However, OSR1 overexpressing animal caps failed

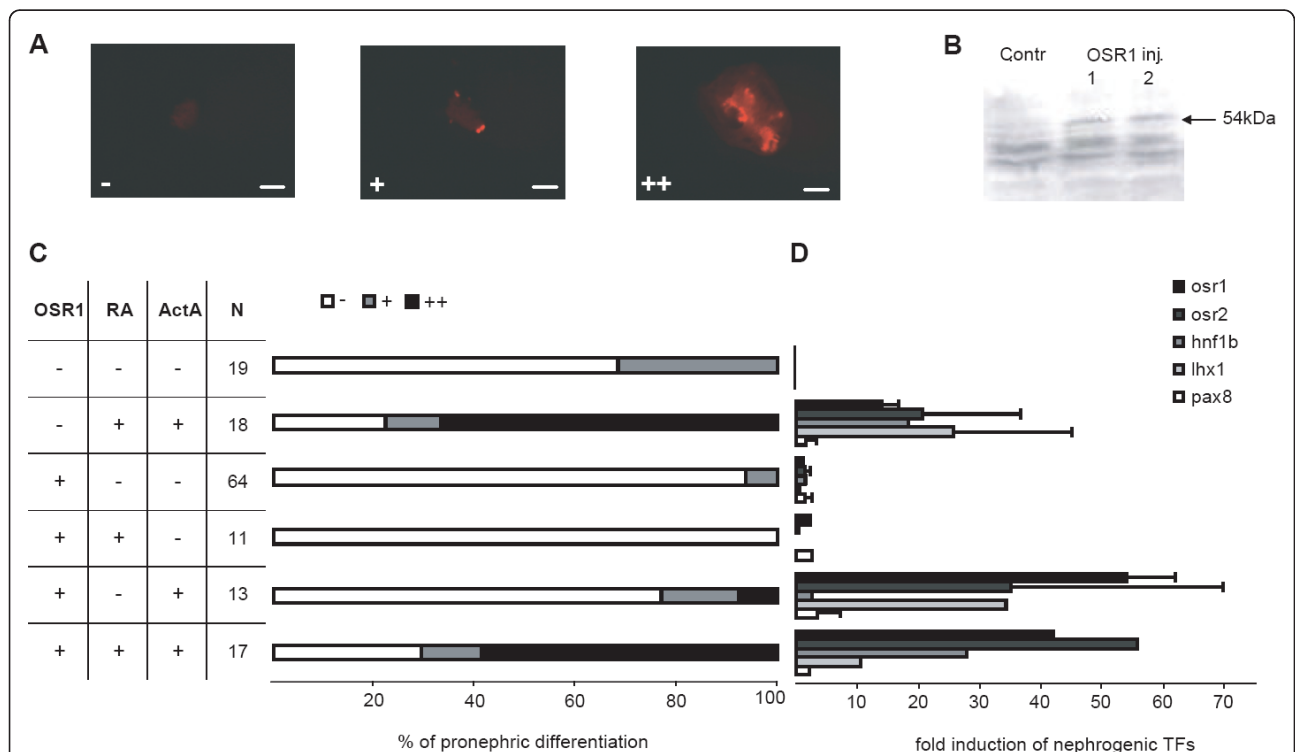


Figure 4 Expression of human OSR1 in animal caps. Animal caps derived from embryos injected with OSR1 mRNA were cultured in Steinberg's solution, retinoic acid (RA, 10⁻⁴M) and/or activin A (ActA, 10 ng/ml) as indicated. To avoid a too high lethality only 150 pg mRNA was injected. **(A)** Classification of whole-mount immunostained animal caps used for the analysis of pronephric tissue induction. The immunostaining was done with a mixture of the antibodies 3G8 and 4A6: - no pronephric tissue, + two or more pronephric cell patches or a small area of pronephric cells, +++ pronephric tubule like structures. Bar = 200 μm **(B)** Exogenous OSR1 is expressed in injected embryos. After cutting the animal caps protein extracts of the remaining late blastula stage embryos injected with OSR1 mRNA were used for Western blot analysis with myc-tag specific antibody. Lane 1 and 2 refer to two pools of ten embryos. The exogenous myc-OSR1 is marked by an arrow. **(C)** The pronephros induction potential in animal caps was assayed after four days using the categories given in panel A. N refers to the number of animal caps investigated. **(D)** Quantitative RT-PCR analysis for the expression of early nephrogenic transcription factors (TFs) of animal caps after three hours and treated as indicated in (C). The bar represents the values of two independent animal cap pools (N = 30) tested. The results of OSR1 injection with retinoic acid treatment alone or in combination with activin A are each from one animal cap pool. Data obtained for *hnf1b* expression were only from one pool tested except for OSR1 injection alone.

to differentiate and this was not improved by adding retinoic acid or activin A. Furthermore, OSR1 overexpression in animal caps treated with retinoic acid and activin A led to a similar extent of pronephric differentiation compared to uninjected, but treated animal caps. Quantitative RT-PCR analysis after three hours showed no induction of the early transcription factors upon OSR1 expression alone or in combination with retinoic acid, whereas treatment of OSR1 injected caps with activin A led to an induction of *osr1*, *osr2* and *lhx1* (Figure 4D), as observed frequently by activin A treatment alone (Figure 1C). As a control we verified the presence of the OSR1 protein translated from the injected OSR1 mRNA by Western blots (Figure 4B). Taken together, OSR1 alone or in combination with retinoic acid or activin A does not have the potential to induce pronephros differentiation in animal caps.

LHX1 and/or PAX8 or HNF1B are not sufficient to induce pronephros differentiation in animal caps

It is known that embryonic overexpression of *Xenopus lhx1* or *pax8* induces enlargement of pronephros and coexpression of both factors leads to a synergistic effect [25]. This enlargement of the pronephros we also observed by unilateral injection of the human transcription factors, since coinjected LHX1 and PAX8 result in a pronephros size of 163 +/- 42% compared to the control side (N = 23, p-value = 3×10^{-7}). However, in contrast to experiments using the *Xenopus* factors [23,25], no ectopic pronephric tissue formation was seen with the human factors. To investigate the pronephros differentiation potential of human LHX1 and PAX8 in animal caps, we injected mRNA encoding these proteins, either alone or in combination, into the animal pole of two-cell stage embryos and analysed the animal caps by immunostaining after four days. Both LHX1 and PAX8 alone or in combination could not induce pronephros differentiation in animal caps at day four (Figure 5A). Measuring the level of the transcripts encoding the five nephrogenic transcription factors after 3 hours we observed no change (data not shown). The missing pronephros formation in animal caps overexpressing LHX1 and PAX8 is consistent with unpublished data reviewed recently [14]. To prove successful injection, we confirmed in all embryos green fluorescence derived from the coinjected GFP mRNA. In addition, for LHX1 injections into animal caps we deduce functional relevant amounts of LHX1 protein, as the *lhx1* target gene *cerberus (cer1)* [38] was 6- or 64-fold induced in two independent experiments (data not shown).

Since differential addition of retinoic acid and activin A to animal caps has shown that activin A alone induces *osr2*, *lhx1* and frequently also *osr1*, but never *hnf1b* that requires retinoic acid in addition (Figure 1C),

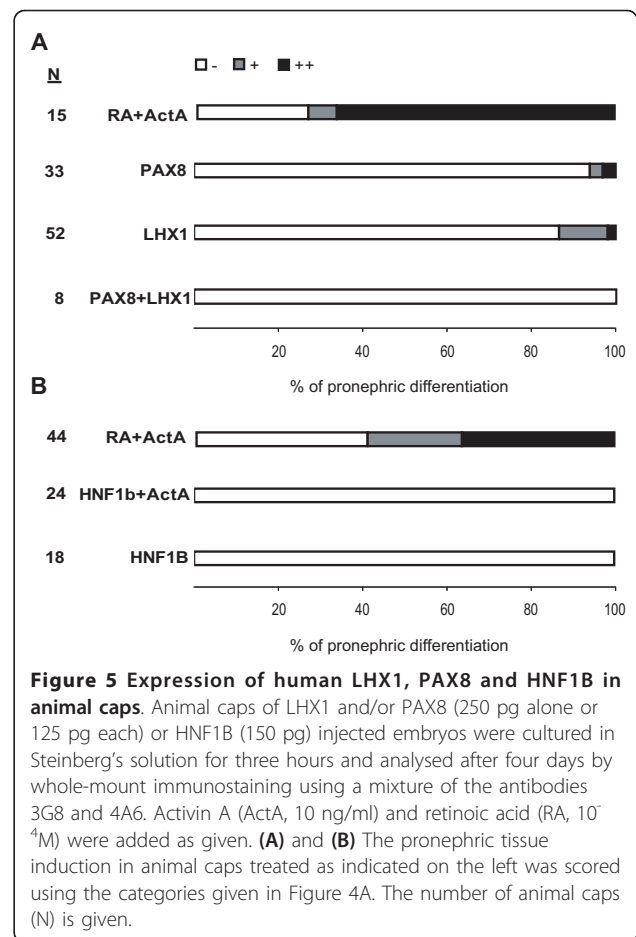


Figure 5 Expression of human LHX1, PAX8 and HNF1B in animal caps. Animal caps of LHX1 and/or PAX8 (250 pg alone or 125 pg each) or HNF1B (150 pg) injected embryos were cultured in Steinberg's solution for three hours and analysed after four days by whole-mount immunostaining using a mixture of the antibodies 3G8 and 4A6. Activin A (ActA, 10 ng/ml) and retinoic acid (RA, 10^{-6} M) were added as given. (A) and (B) The pronephric tissue induction in animal caps treated as indicated on the left was scored using the categories given in Figure 4A. The number of animal caps (N) is given.

we wondered whether HNF1B injection might replace retinoic acid to get pronephric induction in animal caps. However, injection of HNF1B mRNA into the animal pole at the two-cell stage, failed to induce pronephric differentiation in the explanted animal caps, even if cultured in the presence of activin A (Figure 5B). In conclusion, LHX1, PAX8 and HNF1B failed to induce pronephros differentiation in animal caps.

Overexpression of HNF1B induces in animal caps genes important for nephrogenesis

Since numerous target genes of *hnf1b* have been defined [39] or postulated [40] in various mammalian systems, we wonder whether these genes are activated in animal caps representing embryonic stem cells. Thus, we searched for orthologs present in *Xenopus* ESTs and selected 26 genes reported to be expressed early in *Xenopus* development or in the developing pronephros [41]. Analysing animal caps of HNF1B injected embryos by quantitative RT-PCR revealed after seven hours a clear increase in transcripts encoding the early nephrogenic transcription factors *lhx1*, *osr2* and *osr1*, but also of those encoding the transcription factors *hnf1a*, *hnf4a*

Table 1 Activation of genes by HNF1B in animal caps

	gene symbol	fold induction	
		7 h (N = 5)	14 h (N = 4)
early nephrogenic TFs	lhx1	9.9 ↑	1.5
	osr2	4.3 ↑	2.2
	osr1	3.7 ↑	2.5
	pax8	2.4	1.7
	hnf1b	1.3	0.9
genes involved in nephrogenesis	hnf1a	13.7 ↑	7.1
	wnt11b	4.6 ↑	1.3
	wnt11	1.6	1.1
	pax2	1.1	10.7 ↑
	gdnf	1.2 ↑	1.4
	wnt9b	0.8	0.7
proximal tubule genes	prodh2	95.9	1.7
	hnf4a	22.9 ↑	3.4
	anxa13	5.3	1.4
	slc22a6	3.4	25.6
	cpn1	2.3	3.1
	tmem27	2.2	3.5
	slc5a2	1.9	2.3
	tfe3	1.8 ↑	1.2
	rpl35a	1.5	1.5
	gjb1	1.3	1.0
	trps1	1.2	0.8
	ube3a	1.1	0.8
	esd	1.0	1.3 ↑
	rbms1	1.0	0.2 ↓
	slc7a8	1.0	1.5
	fgfr4a	0.9	0.7
	c8a	0.9	1.1
	ncor1	0.9	1.1
	slc4a7	0.9	1.1
	fgfr4c	0.7	0.5 ↓
lhx1 target genes	cer1	421.3 ↑	2.9
	chrd	9.0 ↑	7.8
	pcdh8.2	5.1	2.0
	gsc	3.5	0.1 ↓
	otx2	1.3	1.3

HNF1B (150 pg) injected animal caps were cultured for seven or fourteen hours in Steinberg's solution and analysed by quantitative RT-PCR for gene expression of the early nephrogenic transcription factors, of genes involved in nephrogenesis [78], of proximal tubule genes [40] and of the *lhx1* target genes *chrd* [82], *cer1* [38], *gsc* [82], *otx2* [83] and *pcdh8.2* [84] using the primers given in Additional file 2. Based on the low sample numbers (N = 4 or 5), genes were defined as induced or repressed, if all induction values were either higher or lower for a given probe. Distinct induction or reduction is indicated by an upward or downward arrow, respectively. N is the number of pools with 30 animal caps.

and *tfe3* as well as the signalling molecules *wnt11b* and *gdnf* (Table 1). After fourteen hours we found as a delayed response induction of the transcripts encoding the transcription factor *pax2* and esterase D (*esd*). At this later time point decreased level of transcripts encoding the RNA binding protein *rbms1* and the fibroblast growth factor receptor (*fgfr4c*) were found implying secondary effects. As *lhx1* transcripts are induced by *hnf1b*, we also tested five transcripts of genes known to be targeted by *lhx1*. Indeed, *cerberus* (*cer1*) and *chordin* (*chrd*) were induced, but three other *lhx1* targets were not (Table 1). In fact, *goosecoid* (*gsc*) was downregulated after fourteen hours. Taken together, HNF1B can activate in animal caps several genes involved in kidney development, but some genes considered to be HNF1B targets in mammals are not influenced.

HNF1B regulates *lhx1* transcription by an HNF1 binding site in the *lhx1* promoter

The *Xenopus lhx1* gene is known to be regulated in early embryogenesis by activin A via an activin response element (ARE) present in the first intron [42,43]. Since our data show that HNF1B induces *lhx1* transcripts in animal caps without activin treatment, we explored a direct activation of the *lhx1* gene via HNF1B. By *in silico* analysis with JASPAR [44] we identified potential HNF1 binding sites (Figure 6C) in the promoter region and the first intron of the *Xenopus lhx1* gene (Figure 6A). To determine functional HNF1 binding sites, we tested four luciferase reporter constructs carrying various fragments of the *lhx1* gene (Figure 6A). These constructs were transiently transfected into a HEK293 cell line containing tetracycline-inducible HNF1B [45]. As shown in Figure 6A, the construct Ex-5:B containing the entire *lhx1* gene was inducible by HNF1B and by analysis of various deletion constructs regulation by HNF1B could be pinned down to the HNF1 site of the promoter area (compare Ex1(-120/+3) versus Ex1(-117/+3) in Figure 6A). Clearly, partial mutation (three of twelve base pairs) of the HNF1 site of the promoter (Figure 6C) abolished HNF1B regulation completely. HNF1B transactivation via the HNF1 binding site in the *lhx1* promoter is about 70% (Figure 6A) compared to the effect seen with the HNF4A P2 promoter, a well studied HNF1B target [46,47].

To extend these findings to *Xenopus* embryonic cells we tested some constructs in animal caps that were derived from controls or HNF1B injected eggs. All constructs retaining the HNF1 binding site were transactivated by injected HNF1B, including the minimal construct Ex1(-120/+3), whereas the reporter Ex1(-117/+3) containing

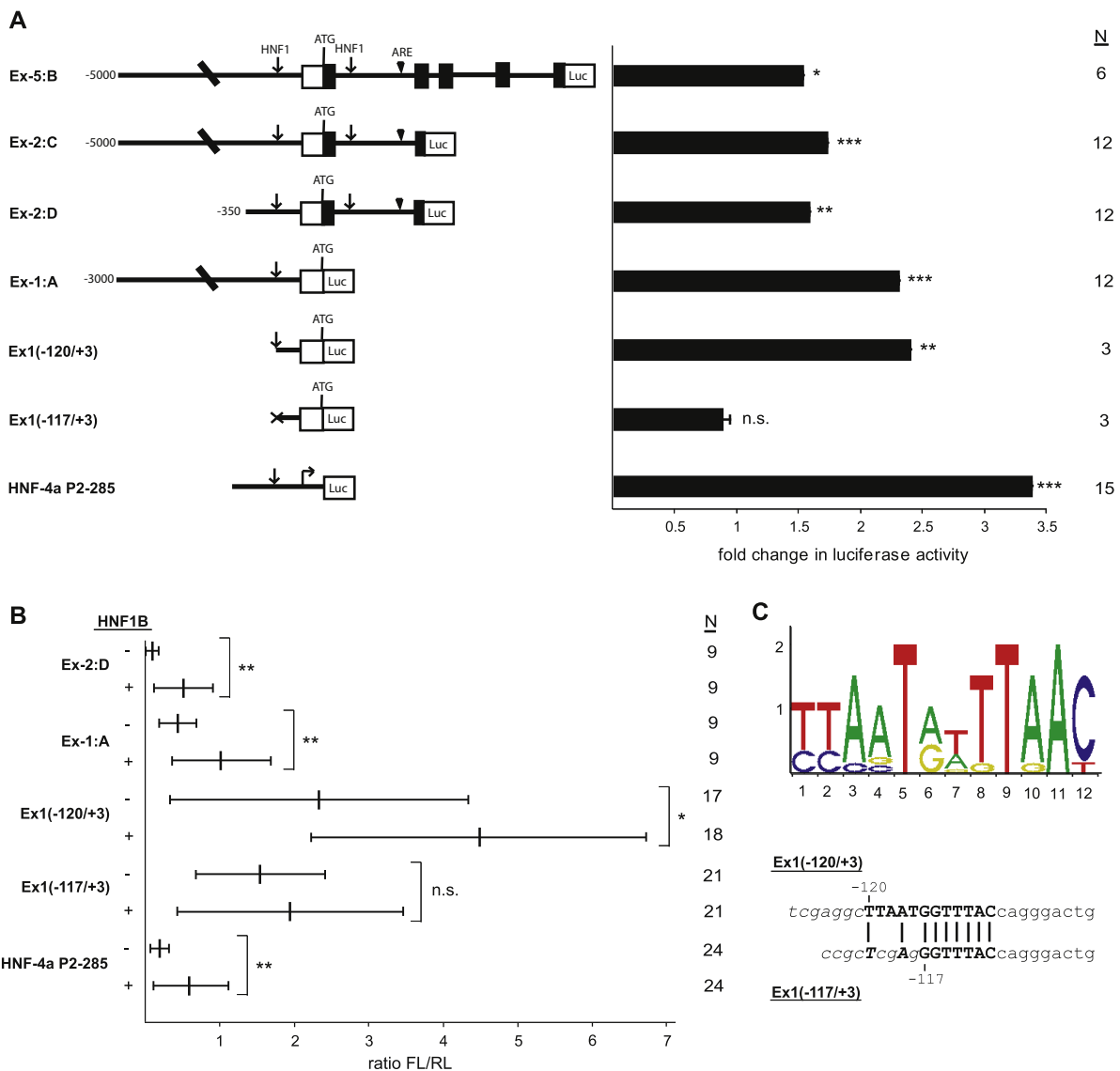


Figure 6 Functional identification of HNF1 binding sites in the promoter region of *lhx1* in HEK293 cells and in animal caps. (A) HEK293 (HNF1B) cells [45] were transfected with *lhx1* gene firefly luciferase fusion constructs and HNF1B expression was induced by adding doxycycline. On the left panel schematic drawings of transfected *lhx1* promoter luciferase fusion constructs (not to scale) are given. Lines represent promoter and intron regions, boxes are exons with protein coding (filled) or untranslated (open) regions (adapted from [42]). Arrows mark potential HNF1 binding sites identified by JASPAR [44]. The arrow head marks the ARE (activin response element) in intron 1 [42]. The Ex1(-120/+3) construct contains the complete HNF1 binding site in the promoter region of *lhx1*, whereas the binding site in the Ex1(-117/+3) construct is partially deleted (compare panel C). The HNF-4a P2-285 construct contains the P2-promoter of the HNF-4a gene which is regulated by HNF1B [46]. The fold change of luciferase activity of transfected constructs in doxycycline induced HEK293(HNF1B) versus untreated cells is given using the *renilla* luciferase reporter pRL-Con as an internal control. The Student's t-test was used and *, ** and *** refer to p-value of ≤ 0.05 , ≤ 0.01 and ≤ 0.001 , respectively and n.s. means not significant. N is the number of independent transfections made. **(B)** The luciferase reporter constructs (50 pg each) were tested in animal caps of controls or HNF1B (150 pg) injected embryos. The luciferase activity was measured in pools of four animal caps after four hours cultivation in Steinberg's solution. To calculate the increase of luciferase activity in HNF1B injected animal caps the ratio of firefly luciferase (FL) to *renilla* luciferase (RL) was used (FL/RL). The vertical line in the middle represents the mean and the small bars the standard deviation. Since experiments with different pools of animal caps were not comparable in quantitative terms, we used the Mann-Whitney-test to score significant differences. * and ** refer to p-value of ≤ 0.05 and ≤ 0.01 , respectively and n.s. means not significant. N is the number of animal cap pools tested. **(C)** The upper panel shows the sequence logo of HNF1 binding site given in JASPAR [44] and the lower panel the sequences of the HNF1 binding site (capital letters) in Ex1(-120/+3) and Ex1(-117/+3) constructs. The vector sequence is indicated by italics.

the partially deleted HNF1 site was not inducible (Figure 6B). From these results we conclude that the *lhx1* promoter carries a functional HNF1 binding site that is active in HEK293 cells as well as in embryonic cells of *Xenopus*.

Discussion

Animal caps are a suitable system to analyse nephrogenesis *in vitro*, because pronephros differentiation can be induced by treatment with activin A and retinoic acid [15]. Activin A simulates as a TGF- β family member the vegetalizing factor 1 (Vg1) [48]. This factor whose maternal mRNA is localized to the vegetal pole of *Xenopus* eggs [49,50] has mesoderm-inducing activity and is an essential regulator of embryonic patterning [51]. On the other hand retinoic acid regulates major embryonic growth and patterning decisions and its availability is regulated by synthesizing and metabolizing enzymes [52]. In *Xenopus* retinal dehydrogenase (RALDH2) creates a critical retinoic acid concentration gradient along the anteroposterior axis [53] and it was shown that retinoic acid treatment of embryos leads to larger pronephros [54], whereas defective retinoic acid signalling impairs pronephros development [55].

Although the animal cap assay represents a powerful system to analyse key molecular events in nephrogenesis, it has some limitations, since pronephros differentiation does not occur in all animal caps. In our hands treated animal caps revealed pronephric induction rate of about 60-85% comparable to about 80% described previously [15,18]. Significantly, animal caps often died during the four day incubation and this lethality was increased upon mRNA injection, but a clear activation of pronephros differentiation was seen in surviving activin A and retinoic acid treated animal caps. The inhomogeneous response of individual animal caps seen by antibody staining was also observed when comparing the induction of specific transcripts between different experiments (Figure 1 and 4). This experimental variation was also evident in transactivation of promoter luciferase reporter constructs (Figure 6B), a limitation reported previously [42,56]. In these experiments the variable outcome is possibly further increased, since reporter constructs and mRNAs cannot be introduced at exactly the same level into each animal cap. In spite of these technical difficulties, we successfully used the animal caps to identify several transcriptional regulatory pathways in this differentiating system.

Our analysis of animal caps treated with activin A and retinoic acid revealed the known induction of *lhx1* (Taira et al., 1992) and *pax8* [22], but also induced expression of *osr1*, *osr2* and *hnf1b* (Figure 1A). Interestingly, in animal caps the kinetics of induction reflects the expression *in vivo*, an observation made for other induced RNAs previously [16,57]. Thus, the expression

of *osr1*, *osr2*, *lhx1* and *hnf1b* after 1.5 hours treatment corresponds with their embryonic expression at early gastrula [19-21] and *pax8* expressed later in animal caps agrees with its expression in late gastrula [25].

In animal caps activin A can induce *osr2* and *lhx1* alone reflecting its strong inducing activity in animal caps [58]. In contrast retinoic acid fails to induce any of the five factors (Figure 1C) correlating the fact that retinoic acid does not induce pronephros [13,59,60]. Previous experiments showed a low *lhx1* induction in retinoic acid treated animal caps [21,61] that possibly reflect subtle differences in the induction protocol or the animals used.

The fast induction of *osr2*, *hnf1b* and *lhx1* reflects most likely a direct activation by activin A and retinoic acid, since the induction of these transcripts was not inhibited by cycloheximide treatment (Figure 1B), a finding that has been previously reported for activin A induced *lhx1* transcripts [21,62]. Consistent with such a direct activation an activin A response element (ARE) has been identified in the *lhx1* promoter [42,43].

Previous experiments showed that it is possible to induce tissue differentiation by overexpressing transcription factors in animal caps. For instance overexpressed *Xbra* leads to mesoderm differentiation with muscle, mesothelium and mesenchyme [63,64], whereas *Sox1* [65] or *Zic3* [66] induce neural tissues. In contrast, overexpression of the five nephrogenic transcription factors failed to trigger pronephros differentiation in animal caps (Figure 4 and 5). However, as we used only the common pronephric markers 4A6 and 3G8, we cannot exclude that some other pronephric differentiation products are induced.

In other experiments the failure of injected transcription factor to induce differentiation in animal caps could be overcome by adding growth factors. For example the induction of neural crest differentiation by *Pax3* and *Zic1* requires Wnt signaling [67] or Neptune induces erythropoiesis only together with GATA1 and bFGF [68]. Concerning pronephros differentiation the ectodermal character of the animal pole cells has possibly first to be changed to a mesodermal fate by adding mesoderm inducing factors. Hence, we added activin A to OSR1 treated animal caps, but without enhancing pronephros differentiation of animal caps (Figure 4C). Since *Osr1* has essential functions at the beginning of kidney development [26,69] and the ability to induce the three early nephrogenic transcription factors *hnf1b*, *lhx1* and *pax8* *in vivo* [19], its inability to induce pronephros differentiation is striking. However, murine cells expressing *Osr1* although multipotent and necessary to build the metanephric precursors require signals from the surrounding tissues for kidney development [70]. Similarly in zebrafish *osr1* is required to limit endoderm

differentiation to allow kidney development [71]. Since OSR1 did not improve the differentiation potential of activin A and retinoic acid treated animal caps (Figure 4C), other signalling molecules are missing. In this context it may be relevant that ectopic kidney tissues in OSR1 (Figure 3B), *osr2* (Tena et al., 2007) or *lhx1* and *pax8* [23,25] overexpressing embryos were found exclusively close to the pronephros. This suggests that signals in the region of pronephros anlage are needed. Most recently *Wnt11b* has been proposed as such a signal [5].

In our experiments we overexpressed either the human or murine transcription factors in order to distinguish the activity of the endogenous gene from the injected RNA, because previous experiments have shown equivalence between *Xenopus* and human *hnf1b*. This we confirmed in principle for OSR1 and *Osr2A* (Figure 3) as well as for coinjected LHX1 and PAX8 (see text). However, in contrast to the *Xenopus* factors, human LHX1 coinjected with PAX8 cannot induce ectopic pronephric tissue and such subtle differences may limit the use of mammalian factors in *Xenopus* embryos.

Investigating the influence of HNF1B on 26 potential *hnf1b* target genes we could show the activation of nine genes in injected animal caps (Table 1). The induced genes include the transcription factor *lhx1*, *hnf1a*, *hnf4a* and *tfe3* suggesting the activation of various transcriptional cascades. This assumption is supported by the observation that the *lhx1* target genes *cer1* [38] and *chrd* [72], both with important roles in nephrogenesis, were also induced. Since other *lhx1* target genes were not activated (Table 1), we assume that some *lhx1* target genes are inhibited by HNF1B expression. It is striking that *cer1* is induced at very high levels and peaked off within seven hours indicating a transient activation of *cer1* by *lhx1* which itself is only transiently induced by HNF1B (Table 1). The activation of *hnf1a* was expected, as HNF1B expression in the *Xenopus* embryos activates *hnf1a* [24] and the *hnf1a* promoter contains a functional HNF1 site [73]. Since HNF1B induces the expression of *osr1* and *osr2* we deduce a positive feedback loop, as *osr1* and *osr2* are able to induce *hnf1b* [19]. Furthermore, an increased expression of the signalling molecules *wnt11b* [5] and *gdnf* [74] were found, both of which play a role in nephrogenesis. Similarly, the downregulation of the fibroblast growth factor receptor (*fgfr4c*) may be relevant, as *fgfr4c* down-regulation is needed to allow pronephros development [75]. The delayed induction of two other important molecules in nephrogenesis, *pax2* [76] and *esd* [77], implied an indirect activation. The fact that several genes crucial for pronephros development and expressed later in more differentiated pronephric tissues were not activated in animal caps by HNF1B, may either reflect that *hnf1b* acts differentially in *Xenopus* compared to

mammals or more likely other signals are missing in the undifferentiated animal cap. Albeit our data show that HNF1B regulates in embryonic cells many genes potentially essential for pronephros development.

A function of *Hnf1b* in early nephrogenesis has most recently been reported also in mice by tetraploid and diploid embryo complementation to overcome early embryonic lethality of homozygous knock-out mice [78]. Significantly, in these mice *Lhx1* was shown to be regulated by *Hnf1b* and to contain an HNF1 binding site in the far upstream promoter. Thus, the regulation of *lhx1* by *hnf1b* seems to be evolutionary conserved, as a 10-fold increase in the expression level of *lhx1* in HNF1B differentiated animal caps is seen (Table 1) and this induction is mediated by an HNF1 binding site in the *Xenopus lhx1* promoter region (Figure 6).

Conclusions

In this study using animal cap assays we provide insight into the network of the early nephrogenic transcription factors in pronephros development and the genes induced by HNF1B, especially (Figure 7). Significantly, we identified a functional HNF1 binding site in the *lhx1* promoter. However, none of the early nephrogenic transcription factors has the ability to induce pronephros differentiation in animal caps.

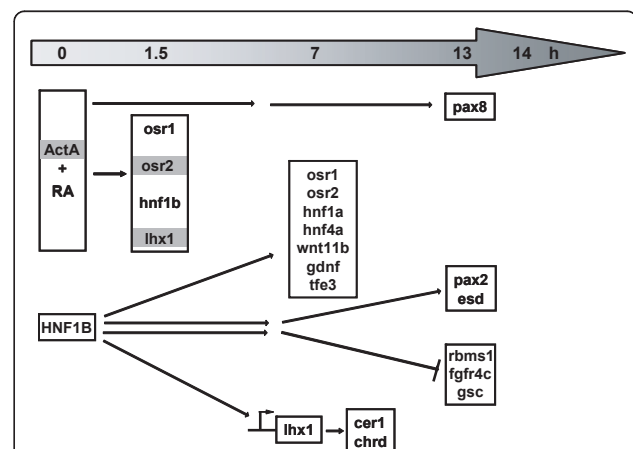


Figure 7 Induction of the early nephrogenic transcription factors and other genes in animal caps. *osr1*, *osr2*, *hnf1b* and *lhx1* are induced already after 1.5 hours treatment with activin A (ActA) and retinoic acid (RA) suggesting that they are direct targets, whereas *pax8* is first expressed after thirteen hours implying an indirect activation. *osr2* and *lhx1* (highlighted in grey) are induced after three hours by activin A alone. HNF1B overexpressed in animal caps induces after seven hours *osr1*, *osr2*, *hnf1a*, *hnf4a*, *wnt11b*, *gdnf* and *tfe3*. After fourteen hours *pax2* and *esd* are increased and *rbms1*, *fgfr4c* and *gsc* are decreased suggesting secondary effects. HNF1B induces the expression of *lhx1* by an HNF1 binding site in its promoter region (arrow). Furthermore, the *lhx1* target genes *cer1* and *chrd* are induced in HNF1B injected animal caps possibly via *lhx1*.

Methods

Plasmids

The myc-Rc/CMVHNF1B expression vector has been described previously [24]. The mouse *Osr2A* plasmid was kindly provided by S. Kawai (Osaka University Graduate School of Dentistry, Osaka, Japan). The full-length open reading frame of the human gene *OSR1* (IRATp970D0444D6; RZPD, Germany) was cloned into pCS2+MT [79] to generate N-terminal myc-tag fusion proteins using 5'-GCTCTAGAGATGGGCAG-CAAAACCTTGCC-3' and 5'-GCTCTAGATTAG-CATTTGATCTTGAGGTTTT-3' as forward and reverse primers, respectively. The *Xba*I restriction sites for cloning are underlined and the construct was verified by sequencing. The full-length open reading frames of the human *PAX8* (RC200651) and *LHX1* (RC210977) in pCMV6 were obtained from OriGene Technologies.

The pRL-Con renilla luciferase construct [80] and the HNF-4a P2-285 pGL3-Basic reporter plasmid [46] have been described. The *lhx1* gene luciferase fusion constructs Ex-1:A, Ex-2:C, Ex-2:D and Ex-5:B [42] were kindly provided by M.L. Rebert (NICHD, USA). The *lhx1* reporter construct harbouring the HNF1 binding site in the promoter region (Ex-2:C) was used for additional constructs. *Xho*I/*Hind*III DNA fragments containing the intact or mutated HNF1 binding site in the promoter region to the transcription start generated by PCR using the primers Ex1(-120/+3): 5'-CCGCTCGAGGCTTAATGGTT-3' (forward), Ex1(-117/+3): 5'-CCGCTCGAGGGTTTACCAG-3' (forward) and 5'-CCCAAGCTTTCCTTTGGTTAT-3' (reverse) were inserted into the *Xho*I and *Hind*III digested pGL3-Basic vector (Promega). The restriction sites for cloning are underlined. Both constructs were verified by sequencing.

mRNA injection

The expression vectors encoding *OSR1*, *Osr2A*, *HNF1B*, *LHX1* and *PAX8* proteins and the GFP encoding expression vector (pCSGFP2) were linearized and *in vitro* transcribed with RNA polymerases (Nielsen and Shaprio, 1986). The restriction enzymes and RNA polymerases used are given in Additional file 1. Capped mRNA encoding the different proteins together with 100 pg of capped green fluorescent protein (GFP) mRNA as internal control were injected into one blastomere of the two-cell stage and after two days, the injected side was scored under a stereofluorescence microscope for the presence of GFP. In case of animal explants, the capped mRNA together with GFP is injected into the animal region of *Xenopus* embryos in each blastomere of the two-cell stage.

Animal cap assays

Xenopus late blastulae, stage 9 [36], were de-jellied by treatment with 2% cysteine hydrochloride in water. The

presumptive ectoderm (animal cap) was isolated with loops of 20 μ m platinum wire heated to about 450°C for a few microseconds using the Gastromaster (Xenotek Engineering, Belleville, USA). The explants were incubated for three hours in Steinberg's solution (58 mM NaCl, 0.67 mM KCl, 0.34 mM Ca(NO₃)₂, 0.83 mM MgSO₄, 3 mM HEPES, pH 7.8) containing recombinant human activin A (10 ng/ml; Sigma, A4941) and all-trans retinoic acid (10⁻⁴M; Sigma, R2625) or only in Steinberg's solution for controls and for explants from mRNA injected embryos. After three-times washing with Steinberg's solution the explants were cultured in Steinberg's solution at 20°C until they were equivalent to stage 40-42 in normal embryos (four days) and used for whole-mount immunostaining.

Quantitative RT-PCR

RNA from pools of 30 animal caps was isolated with peqGold RNAPure (PiqLab) followed by phenol/chloroform extraction. For cDNA synthesis the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used. SYBR-Green real-time PCR was performed on a 7900HT Sequence Detection System (Applied Biosystems) using Power-SYBRGreen Mix (Applied Biosystems). Templates were determined in duplicate and for primers used see Additional file 2. Results are normalized to ornithin decarboxylase (*odc*) expression levels. In all cases water only and reverse transcriptase negative controls failed to produce specific products. The fold induction of the early transcription factors was obtained by comparison of treated and untreated animal caps.

Whole-mount immunostaining

Whole-mount immunostaining of four day cultured animal caps or animals at the swimming larval stage were done as described [81]. The difference between the injected and the non-injected sides of embryos was evaluated by measuring the whole area using the lateral view with the widest diameter from the dorsal to the ventral side of the immunostained pronephros including the pronephric tubules and the anterior part of the pronephric duct. The measurements were made using Axio-Vision 4.6 software (Carl Zeiss Imaging Solutions), and the non-injected side was used as a reference for each animal. The values representing kidney size obtained from each mRNA injected embryo were compared to values obtained from GFP control-injected embryos (data adapted from [23]). Significant differences were scored using the Student's *t*-test to calculate *p*-values.

Cell culture and transient transfection assays

The HEK293 (*HNF1B*) cell line [45] contains a tetracycline-inducible *HNF1B* transgene. In a 96-well plate (17,500 cells/well) 30 ng of the promoter constructs

were cotransfected using FuGeneHD (Roche) with 0.05 ng of renilla luciferase plasmid pRL-Con for normalization of transfection efficiencies. Four hours after transfection HNF1B expression was induced by the addition of 1 µg/ml doxycycline. Twenty-four hours after transfection firefly and renilla luciferase activities were measured in triplicate with the Dual-Luciferase Reporter Assay (Promega).

Luciferase reporter assays in animal caps

50 pg reporter constructs and 150 pg HNF1B mRNA were coinjected into the animal region of *Xenopus* embryos at the two-cell stage together with renilla luciferase as internal control. Animal caps were cut, cultured in Steinberg's solution for four hours and pools of four caps were assayed by the Dual-Luciferase Reporter Assay (Promega). As the absolute levels of luciferase activity varied between pools of animal caps (data not shown, also described previously [42]), the Mann-Whitney-test was used to score significant differences.

Additional material

Additional file 1: Table S1: Expression vectors with restriction enzymes and RNA polymerases used for RNA synthesis.

Additional file 2: Table S2: List of primers used for quantitative RT-PCR.

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Authors' contributions

CD carried out all the experiments with the animal caps and the injected embryos and drafted the manuscript. SS made the transfection assays in cell culture. GUR conceived the study, participated in its design and coordination and helped to finish the manuscript. All authors read and approved the final manuscript.

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