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Lin28 proteins are required for germ layer specification in *Xenopus*

Laura Faas, Fiona C. Warrander, Richard Maguire, Simon A. Ramsbottom, Diana Quinn, Paul Genever and Harry V. Isaacs*

SUMMARY

Lin28 family proteins share a unique structure, with both zinc knuckle and cold shock RNA-binding domains, and were originally identified as regulators of developmental timing in *Caenorhabditis elegans*. They have since been implicated as regulators of pluripotency in mammalian stem cells in culture. Using *Xenopus tropicalis*, we have undertaken the first analysis of the effects on the early development of a vertebrate embryo resulting from global inhibition of the Lin28 family. The *Xenopus* genome contains two Lin28-related genes, *lin28a* and *lin28b*. *lin28a* is expressed zygotically, whereas *lin28b* is expressed both zygotically and maternally. Both *lin28a* and *lin28b* are expressed in pluripotent cells of the *Xenopus* embryo and are enriched in cells that respond to mesoderm-inducing signals. The development of axial and paraxial mesoderm is severely abnormal in lin28 knockdown (morphant) embryos. In culture, the ability of pluripotent cells from the embryo to respond to the FGF and activin/nodal-like mesoderm-inducing pathways is compromised following inhibition of lin28 function. Furthermore, there are complex effects on the temporal regulation of, and the responses to, mesoderm-inducing signals in lin28 morphant embryos. We provide evidence that *Xenopus* lin28 proteins play a key role in choreographing the responses of pluripotent cells in the early embryo to the signals that regulate germ layer specification, and that this early function is probably independent of the recognised role of Lin28 proteins in negatively regulating let-7 miRNA biogenesis.

KEY WORDS: *lin28a*, *lin28b*, *Xenopus*, Mesoderm, miRNA, let-7, Pluripotency, Germ layer, FGF, Activin, Nodal

INTRODUCTION

The RNA-binding protein LIN-28 was originally identified in *Caenorhabditis elegans* as a regulator of timing during development (Ambros and Horvitz, 1984; Moss et al., 1997). Lin28 family proteins contain a unique combination of both zinc knuckle and cold shock RNA-binding domains that has been conserved during evolution, and Lin28 proteins are present in a wide range of animal groups (Moss and Tang, 2003).

There is considerable interest in the function of Lin28 proteins as regulators of pluripotency in stem cells. LIN28A is expressed at high levels in proliferative, pluripotent embryonic stem (ES) cells, but is downregulated during human and murine ES cell differentiation (Darr and Benvenisty, 2008; Viswanathan et al., 2008). Furthermore, LIN28A, in combination with NANOG, OCT4 and SOX2, can reprogram somatic cells to a pluripotent ES cell phenotype (Yu et al., 2007). Lin28 proteins are involved in post-transcriptional regulation via direct association with target mRNAs (Polesskaya et al., 2007; Xu and Huang, 2009; Qiu et al., 2010; Jin et al., 2011; Peng et al., 2011; Lei et al., 2012) and recent studies show their role in negatively regulating the biogenesis of mature let-7 family miRNAs (Heo et al., 2008; Piskounova et al., 2008; Viswanathan et al., 2008; Hagan et al., 2009; Nam et al., 2011). let-7 miRNA expression levels generally correlate with the differentiated state of cells, and low let-7 expression has been linked with poor prognosis in several cancer types (Boyerinas et al., 2010). A prevailing model suggests that the balance between the levels of Lin28 proteins and let-7 miRNAs is important for the self-renewal and differentiation of ES cells (Viswanathan and Daley, 2010). However, a recent study indicates that LIN28A is not required for the self-renewal of ES cells (Darr and Benvenisty, 2008). At present, the exact role of Lin28 proteins in stem cell regulation is unclear.

Genome-wide association studies have also implicated *Lin28* genes as regulators of growth and timing of developmental events in humans (Lettre et al., 2008; Hartge, 2009; Viswanathan and Daley, 2010). Analysis of LIN28A-overexpressing mice indicates that these roles are conserved in other vertebrate species (Zhu et al., 2010). However, most of the evidence available at present is focused on processes in later development, and the role of Lin28 proteins in early development, at the time when the main body is established, remains elusive.

Here, we investigate the function of *lin28a* and *lin28b* in the early development of the amphibian *Xenopus*, a well-established model for understanding the role of growth factors in regulating lineage restriction during vertebrate development. We show that *lin28a* and *lin28b* have overlapping, but distinct, expression profiles. Notably, lin28b protein is present maternally in the early embryo, whereas lin28a protein is only present after the activation of the zygotic genome at the mid-blastula transition (MBT). *lin28a* and *lin28b* are expressed in the pluripotent cells of the animal hemisphere and are enriched in the cells of the early mesoderm. Our data suggest a role for fibroblast growth factors (FGFs) in regulating the expression of *lin28* genes within the mesoderm.

In addition, individual and compound knockdowns of the three *Xenopus* lin28 proteins lead to dramatic inhibition of dorsal development that is characterised by greatly reduced development of axial and paraxial mesoderm. We show that knockdown of lin28 function in pluripotent cells of the early embryo compromises their ability to respond appropriately to mesoderm-inducing growth factors. Furthermore, lin28 knockdown leads to shifts in the temporal expression profiles of a number of key genes involved in

Area 11, Department of Biology, University of York, York, YO10 5DD, UK.

*Author for correspondence (harry.isaacs@york.ac.uk)

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mesoderm specification. This represents the first evidence that Lin28 family genes play a key role in regulating the timing of, and responses to, growth factor signalling in the early development of a vertebrate embryo.

A key finding of this study is that, although amphibian lin28 proteins exhibit let-7 miRNA-binding activity, no significant effects on the abundance of let-7a, let-7f and let-7g miRNAs are detected in lin28 knockdown embryos, at the stage when germ layer specification occurs. Our data indicate that the requirement for lin28 function in the initial specification of the mesoderm is likely to be independent of its role in let-7 family biogenesis. By contrast, lin28 function seems to be required to regulate let-7 levels at later stages, after germ layer specification, indicating differential roles for lin28 in amphibian development: a let-7-independent early role and a let-7-dependent later role.

MATERIALS AND METHODS

Embryo methods

X. tropicalis embryos were produced as previously described (Khokha et al., 2005; Winterbottom et al., 2010). Embryos were injected at the 2- or 4-cell stage and cultured at 22°C. Animal cap explants were dissected at Nieuwkoop and Faber (NF) stage 8 (Nieuwkoop and Faber, 1994) and treated with 10 U/ml recombinant FGF4 protein (Isaacs et al., 1992) or 5 U/ml recombinant murine activin (Sigma).

mRNAs

mRNA was synthesised as described previously (Branney et al., 2009). The dominant-negative *X. laevis* FGFR4a (dnFGFR4) plasmid was a gift from Harumasa Okamoto (Hongo et al., 1999). Constructs coding for C-terminal HA epitope-tagged lin28 proteins were generated by PCR using reverse primers including a sequence coding for the YPYDVPDYA peptide. Subclones of the coding region of each lin28 isoform lacking both 5′ and 3′ UTRs were generated by PCR.

Morpholino antisense oligonucleotides (MOs)

MOs were synthesized by Gene Tools. Standard control MO, 5′-CCTCCTACCTCAGTTACAATTTATA-3′; lin28a1 MO, 5′-GGTCTGCC-TTGAAGTTGTCCCAGCT-3′; lin28a2 MO, 5′-GAGTCTCTTCTA-TCTGAGGTCAGGC-3′; lin28b MO, 5′-TCCTTCGGCCATGATGCC-TCCTGCT-3′.

miRNA duplexes

The let-7 duplex and mutant let-7 duplex sequences injected have been reported previously (Kloosterman et al., 2004).

In situ hybridisation

DIG-labelled probes were synthesized using DIG Labeling Mix (Roche Diagnostics). *In situ* hybridisation was performed as previously described (Harland, 1991; Reece-Hoyes et al., 2002).

Western blots

Western blots were carried out as described previously (Winterbottom et al., 2010). Affinity-purified *X. tropicalis* anti-lin28 antisera were produced by Enzo Life Sciences (Exeter, UK) in rabbits by inoculation of peptides corresponding to the C-terminal sequences of *X. tropicalis* lin28a1/a2 (EEQPISEEQELIPETME) or lin28b (SRKGPSVQKRKKT) proteins. Antibody dilutions were as follows: anti-lin28a and anti-lin28b, 1:1000; anti-HA (Sigma), 1:5000; anti-ERK1/2 (Sigma), 1:100,000; anti-dpERK (Sigma), 1:4000; anti-p-Smad2 (Millipore), 1:1000; anti-GAPDH (Santa Cruz), 1:2000; anti-mouse POD (Amersham Biosciences), 1:5000; anti-rabbit POD (Amersham Biosciences), 1:10,000. POD detection was carried out using the BM Chemiluminescence Blotting Substrate (Roche) and ECL Hyperfilm (Amersham).

Immunofluorescence

Cryosectioned embryos were processed for immunohistochemistry as described (Roth et al., 2010), with the following modifications: prior to sectioning, embryos fixed in MEMFA for 1 hour at room temperature were transferred to 15% fish gelatine/15% sucrose for 16 hours followed by a second 16-hour incubation in 25% fish gelatine/15% sucrose. Embryos were then sectioned at 15 μm. Lin28a and lin28b rabbit polyclonal antibodies were used at 1:200. Goat anti-rabbit Alexa Fluor 488 (Invitrogen, A11034) was used at 1:250. Once processed, slides were mounted in Vectashield mounting medium with DAPI (Vector Labs, H-1200). Peptide competition assays were carried out using the relevant epitope peptides at 1 μg/ml for 45 minutes at room temperature. Fluorescence was imaged on a Zeiss LSM 710 confocal microscope mounted on an Axio Observer.Z1 inverted stage.

Quantitative PCR (Q-PCR) for mRNA expression

Total RNA was isolated using TRI Reagent (Sigma). cDNA was synthesised using SuperScript II reverse transcriptase and oligo(dT) primers (Invitrogen). Q-PCR reactions were carried out in triplicate on an ABI Prism 7300 detection system (Applied Biosystems) using SYBR Green I PCR Master Mix (Applied Biosystems). Relative expression levels of each gene were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) and normalised to *ornithine decarboxylase* (*ODC*).

Q-PCR for miRNA expression

Total RNA was isolated using miRVana miRNA Isolation Kit (Applied Biosystems). cDNA was synthesized with miRNA-specific primers for TaqMan assays (Applied Biosystems) using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems). Q-PCR reactions were carried out in quadruplicate using TaqMan Universal Master Mix II (Applied Biosystems) with TaqMan miRNA probes (Applied Biosystems). miRNA expression levels were normalised to *U6*.

RNA electrophoretic mobility shift assay (EMSA)

RNA oligonucleotides (2 μM) were labelled using the KinaseMax Kit (Applied Biosystems). Binding reactions were performed at room temperature in 10 μl total volume (Piskounova et al., 2008), with 0.5 μl labelled probe and diluted embryo extract in lysis buffer comprising 50 mM Tris pH8, 25% glycerol, 50 mM KCl, 2 mM DTT, 0.1 mM EDTA and Proteinase Inhibitor Cocktail III (Calbiochem). Binding buffer comprised 60 mM KCl, 10 mM HEPES pH 7.6, 3 mM $MgCl_2$, 5% glycerol, 1 mM DTT, 5 μg/μl heparin and 150 ng yeast tRNA/reaction. Twenty units of RNasin (Promega) were added per binding reaction with embryo lysates. Binding reactions were resolved on 8-10% polyacrylamide native gels. Supershifts were carried out using a 1:20 dilution of custom lin28a or lin28b antiserum (Enzo Life Sciences), or the corresponding pre-immune bleed (serum control) per binding reaction. Antiserum was pre-incubated with the protein sample and binding buffer for 20 minutes on ice, before labelled probe was added for 20 minutes at room temperature. The sequence of the let-7g terminal loop is: 5′-UUUGAGGGUCUAUGAUACCACCCGGUAC-AGGAGAU-3′.

RESULTS

Identification of *X. tropicalis lin28a* and *lin28b*

A previous study showed that a *lin28a* orthologue is present in *Xenopus laevis* (Moss and Tang, 2003). Analysis of Lin28 family cDNAs in the closely related diploid species *X. tropicalis* identified one *lin28b* orthologue and two sequences closely related to *lin28a*. Alignments of the *X. tropicalis* lin28a and lin28b protein sequences are shown in supplementary material Fig. S1A. Analysis of the *lin28a* genomic locus indicates that the two *lin28a*-related sequences, which we have designated *lin28a1* and *lin28a2*, result from alternative splicing of two small exons coding for different N-termini (supplementary material Fig. S1B).

Expression of *X. tropicalis lin28a* and *lin28b*

Previous studies showed that *Lin28a* mRNA and protein are expressed widely in all three germ layers during early development of mouse and chicken (Yang and Moss, 2003; Yokoyama et al., 2008). In *Xenopus*, most zygotic gene expression commences after the MBT.

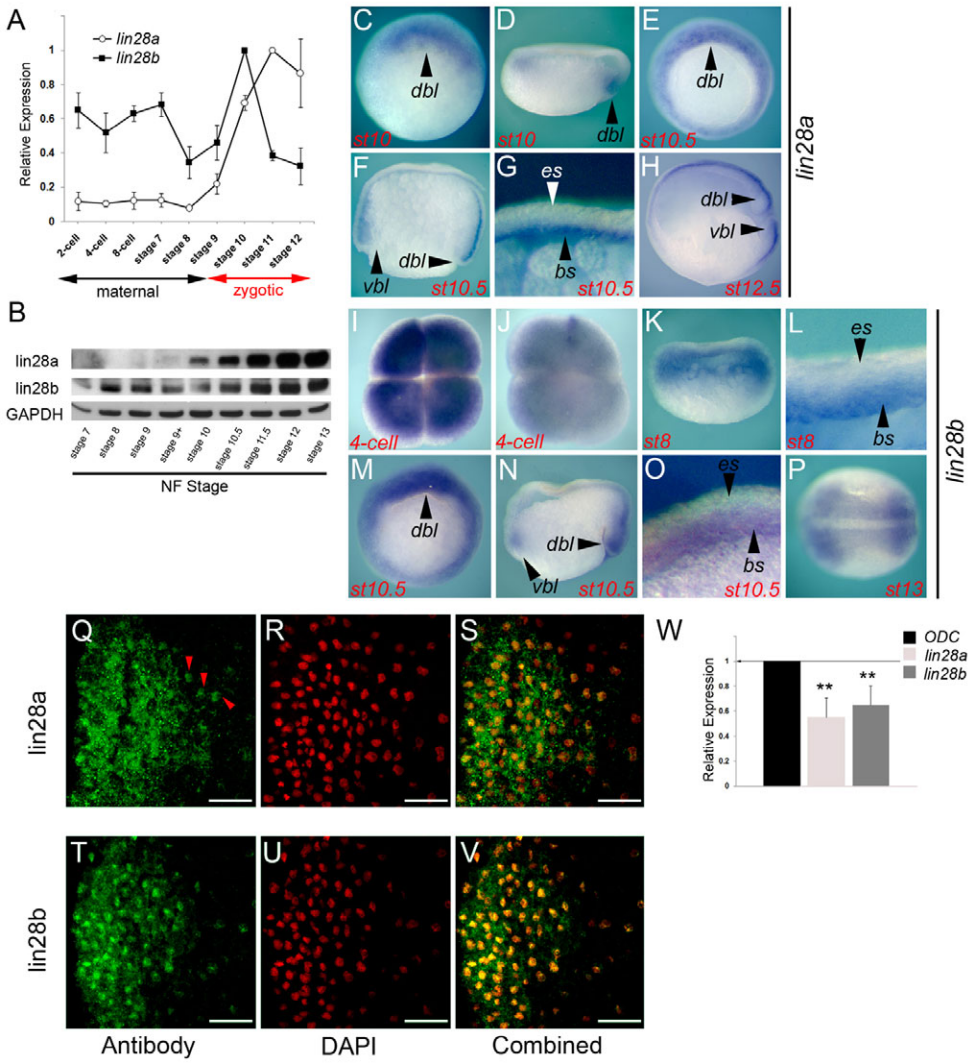

Fig. 1. Expression of *lin28a* and *lin28b* in the *X. tropicalis* embryo. (**A**,**B**) Temporal expression profiles of *lin28a* and *lin28b* mRNA (A) and protein (B) in early cleavage to late gastrula stage embryos as assessed by Q-PCR and western blot, respectively. (**C-P**) Spatial expression of *lin28a* (C-H) and *lin28b* (I-P) as assessed by *in situ* hybridisation. (C,E,M) Vegetal view; (D,F,H,K,N) transverse sections, lateral view; (G,L,O) higher magnification of F, K and N, respectively; (J) vegetal view of I. Stages are indicated: stage 8, mid-blastula; stage 10 to 10.5, early gastrula; stage 12.5 to 13, late gastrula. *dbl*, dorsal blastopore lip; *vbl*, ventral blastopore lip; *es*, ectodermal superficial layer; *bs*, basal sensorial layer. (**Q-V**) Fluorescence microscopy of cryosections from the circumblastoporal region of early gastrula stage 10.5 embryos. (Q) Immunolocalisation of lin28a expression. Red arrows indicate nuclear enrichment. (R) Nuclear DAPI staining in same section as Q. (S) Merge of Q and R. (T) Immunolocalisation of lin28b expression. (U) Nuclear DAPI staining in same section as T. (V) Merge of T and U. (**W**) Expression levels of *lin28a*, *lin28b* and the ubiquitously expressed housekeeping gene *ornithine decarboxylase* (*ODC*) in dnFGR4-injected embryos as compared with control embryos by Q-PCR. Solid line represents mRNA levels in corresponding control embryos (set at 1). ***P*<0.005 (Student's *t*-test), for expression in experimental embryos compared with control embryos. Error bars indicate s.d. Scale bars: 50 μm.

Transcription of *lin28a* begins shortly after the MBT (Fig. 1A); before the MBT there is only a low level of maternally deposited *lin28a* mRNA present. The level of *lin28b* mRNA also begins to rise after the MBT, but in contrast to *lin28a* there is a relatively high level of maternal *lin28b* mRNA. In keeping with these data, western blot analyses show that low levels of lin28a protein are detected just after the MBT at blastula stage 9+. By the start of gastrulation at stage 10, the level of lin28a protein has risen significantly, and continues to rise through the gastrula stages (Fig. 1B). Maternally deposited lin28b is detected in pre-MBT stage embryos and protein levels remain constant through late blastula and gastrula stages.

Analysis of *lin28* mRNA localisation reveals that maternal *lin28b* mRNA is enriched in the animal hemisphere relative to the vegetal hemisphere (Fig. 1I,J). Animal localisation of *lin28b* persists in the deep sensorial layer of the presumptive ectoderm into blastula stages (Fig. 1K,L). The initial zygotic expression of *lin28a* and *lin28b* is enriched in the presumptive mesoderm of the circumblastoporal region of the embryo (Fig. 1C,E,M). Both genes show a marked dorsal to ventral expression gradient within the mesoderm (Fig. 1C,D,M,N). Expression of both genes persists in the deep sensorial cell layer of the animal hemisphere during gastrula stages (Fig. 1F,G,O).

By the end of gastrulation *lin28a* is expressed around the closed blastopore, with dorsal expression in the ectoderm extending further toward the anterior of the embryo than on the ventral side (Fig. 1H). *lin28b* expression is localised to the dorsal side of the embryo and is enriched at both the anterior and posterior ends of the neural plate (Fig. 1P).

Differential subcellular localisation of lin28a and lin28b proteins

It has recently been shown that mammalian Lin28a and Lin28b localise to different subcellular compartments and that this differential localisation in part accounts for the dissimilar modes of action of the two proteins; Lin28a protein is found mainly in the cytoplasm whereas Lin28b is highly enriched in the nucleus of cells in culture (Piskounova et al., 2011). We investigated whether amphibian lin28 proteins are also differentially localised within the cells of the early embryo. Immunofluorescence shows that lin28a and lin28b are localised in cells of the circumblastoporal region of the *Xenopus* embryo at the start of gastrulation (Fig. 1Q-V). Moreover, control experiments indicate that the observed immunofluorescence is specific for each of the proteins; the immunoreactivity can be effectively competed out by the relevant peptide immunogens (supplementary material Fig. S2).

Lin28a immunoreactivity is present throughout the cytoplasm of most cells and is also commonly seen in bright cytoplasmic puncta (Fig. 1Q-S). However, lin28a protein does not appear to be excluded from the nucleus, and in some cells (red arrows, Fig. 1Q) immunoreactivity is enriched in the nucleus. By contrast, immunoreactivity for lin28b is enriched in the nuclei of most expressing cells, but is also found at lower levels in the cytoplasm (Fig. 1T-V). We note that the bright, punctate, cytoplasmic fluorescence observed with lin28a is less common with lin28b.

FGF signalling and the regulation of *lin28* expression

Our previous study showed that *X. laevis lin28a* is downregulated in response to global inhibition of FGF signalling (Branney et al., 2009), highlighting lin28a as a component of a putative FGF-regulated pathway operating during the process of germ layer specification. Here we show that the levels of both *lin28a* and *lin28b* mRNAs are significantly downregulated in *X. tropicalis* gastrula stage embryos in response to FGF inhibition with a dominant-negative FGF receptor (Fig. 1W), indicating that FGF signalling is required for normal *lin28* expression during *Xenopus* development. Furthermore, our observation that *lin28* expression is enriched in the early mesoderm is in keeping with a role for FGF in regulating normal *lin28* expression because the early mesoderm represents a key domain of FGF activity in the early embryo (Christen and Slack, 1999; Branney et al., 2009).

Effects of lin28a and lin28b inhibition

To gain insight into lin28 function during development, we investigated the effects of overexpressing or knocking down lin28 proteins in the early embryo. Injection of up to 1 ng of synthetic *lin28a* or *lin28b* mRNAs has no gross phenotypic effects on the development of *X. tropicalis* embryos (data not shown). By contrast, single or compound knockdowns of lin28a1, lin28a2 and lin28b proteins using translation-blocking MOs produce a spectrum of phenotypic effects ranging from mild inhibition of dorso-anterior development, including loss of eyes and head structures, to a severe shortening of the dorsal axis (Fig. 2A,B; supplementary material Fig. S3A,C-F). The highest proportion of severe phenotypes is observed in the lin28a1, lin28a2 + lin28b (all lin28 MOs) compound knockdown embryos (Fig. 2B). Lin28 MOs used in this study effectively inhibit the translation of epitope-tagged lin28 proteins encoded by synthetic mRNAs containing the respective MO target sequences (supplementary material Fig. S3B), as well as the endogenous lin28a and lin28b proteins (Fig. 2C,D). Moreover, the observed effects are specific, as the phenotypic effects of lin28 knockdown are rescued by co-injection of non-targetable *lin28* mRNAs (supplementary material Fig. S3A,C-I).

Histological analysis of lin28 compound knockdown (morphant) embryos shows that the organisation of tissues in the dorsal axis is disturbed compared with controls (Fig. 2E,G), and that these effects are not restricted to a single germ layer. Thus, the patterning of the ectodermally derived brain and neural tube is highly abnormal in morphants (Fig. 2E,F), and, in keeping with the observed inhibited axial elongation, notochord and skeletal muscle tissue is reduced, indicating that the development of the dorsal axial and paraxial/somitic mesoderm is also abnormal (Fig. 2E-H).

Lin28 and the expression of genes in the early mesoderm

Given the observed effects of lin28 knockdown on the differentiation and patterning of mesodermal structures at larval stages, we were interested to see whether these late effects might result from prior effects on lin28 function in the early mesoderm, which, as discussed, is a site of zygotic *lin28a* and *lin28b* expression. Effects on the levels of a number of key genes expressed in, and required for, the specification of the mesoderm were analysed in lin28 morphant embryos at early gastrula stages.

Xenopus brachyury (Xbra) is a T-box transcription factor that is widely expressed in the early mesoderm (Smith et al., 1991). Chordin is a secreted BMP inhibitor expressed in the presumptive dorsal axial mesoderm (Sasai et al., 1994; Holley et al., 1995). MyoD is a bHLH transcription factor expressed in the myogenic lineage within the paraxial mesoderm from early gastrula stages (Hopwood et al., 1992). *In situ* hybridisation analysis shows that the levels of expression and size of the expression domains of the *Xbra*, *chordin* and *myoD* genes are greatly reduced in lin28 morphants (Fig. 3A). By contrast, no effects on the expression domain of the early endoderm marker *Sox17b* are observed in morphants (Fig. 3A).

Further quantitative analysis of gene expression levels in morphant embryos reveals significant inhibition of several genes expressed in the very early mesoderm, ranging from a 35% reduction for *mesogenin* to 80% reduction for *Tbox6* (Fig. 3B).

Lin28 knockdown alters the response of pluripotent cells to mesoderm-inducing signals

The FGF pathway is required for the normal development of the vertebrate mesoderm (Dorey and Amaya, 2010; Pownall and Isaacs, 2010). Our observation that *lin28* expression is reduced in response to FGF inhibition and that *lin28* genes are expressed in tissues that respond to FGF prompted us to investigate the effects of lin28 knockdown on FGF-mediated mesoderm induction.

The cells of the amphibian animal hemisphere constitute a pluripotent stem cell population that, upon exposure to appropriate growth factor signals, can be induced to form all three germ layers (De Robertis, 2006; Heasman, 2006). Blastula stage explants of animal hemisphere cells, known as animal caps, cultured in isolation differentiate as masses of atypical epidermis. However, when cultured in the presence of FGF these same cells respond by differentiating as a range of mesodermal tissue types (Slack et al., 1987; Isaacs, 1997). Fig. 4A shows control animal caps after 3 days in culture with or without FGF4 protein. The untreated control animal caps typically form rounded masses of tissue, which histology reveals as consisting of unlayered, atypical epidermis (Fig. 4B). By contrast, culture in the presence of FGF induces the formation of fluid-filled vesicles that are surrounded by epidermis but contain a range of mesodermal tissues, including mesenchyme and mesothelium (smooth muscle) (Fig. 4A,B). When the same

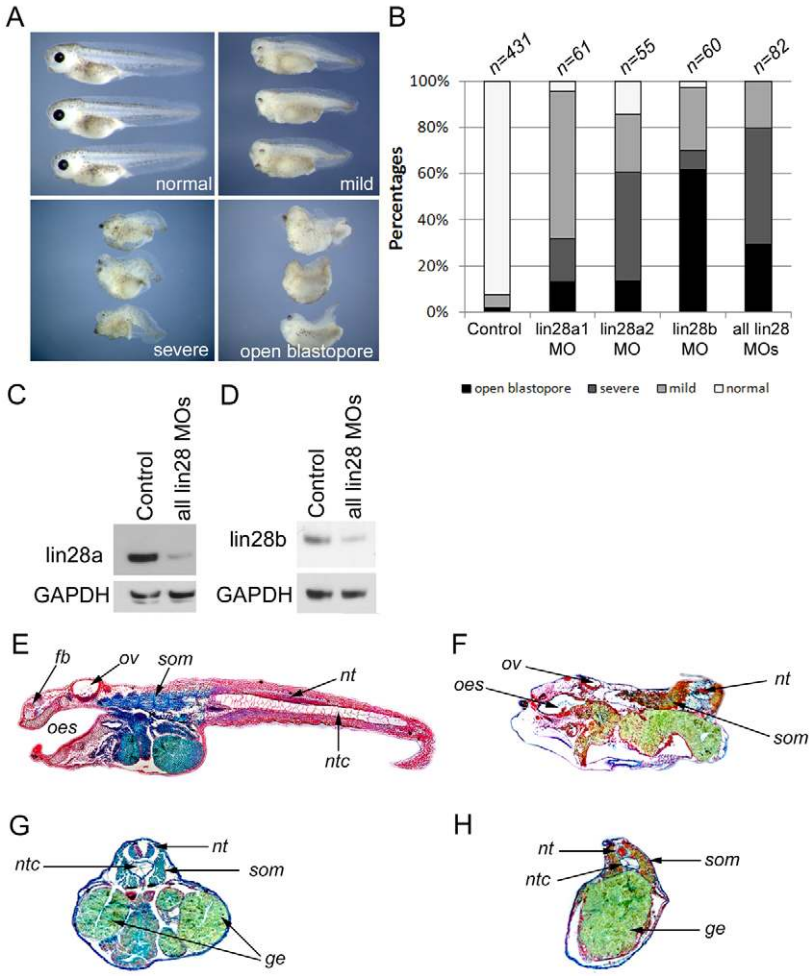

Fig. 2. Phenotype of lin28 morphants. (**A-D**) Range of axis defects in *X. tropicalis* lin28 morphants, from unaffected ('normal') to open blastopore. (B) Percentage of phenotypes in lin28a1, lin28a2, lin28b and all lin28 (i.e. in28a1 + lin28a2 + lin28b) morphant embryos. (C,D) Depletion of lin28a (C) and lin28b (D) proteins in lin28 (lin28a1 + lin28a2 + lin28b) morphant embryos as assessed by western blot at stage 20. GAPDH, loading control. (**E-H**) Histology on sagittal (E,F) and transverse (G,H) sections of lin28 morphants (F,H) and controls (E,G). *fb*, forebrain; *ge*, gut endoderm; *nt*, neural tube; *ntc*, notochord; *oes*, oesophagus; *ov*, otic vesicle; *som*, somites.

experiment is undertaken with animal caps from lin28 morphant embryos, the appearance of explant cultures in isolation is broadly unchanged. However, lin28 knockdown greatly inhibits the formation of fluid-filled vesicles and the differentiation of mesoderm following culture in the presence of FGF (Fig. 4A,B), indicating that lin28 function is required for animal caps to respond appropriately to FGF signalling.

Current models suggest that FGFs are not the primary signals for mesoderm specification in amphibians, but are secondary signals produced in the nascent mesoderm in response to the primary signals, such as nodal and activin (Isaacs et al., 1994; Schulte-Merker and Smith, 1995; Isaacs, 1997). We therefore investigated whether lin28 knockdown also interferes with the ability of activin to induce mesoderm and gastrulation-like elongation in animal cap explants. Similarly, we found that lin28 knockdown blocks the elongation of animal hemisphere explants and the differentiation of mesoderm induced by activin (Fig. 4C,D).

Thus far, we have shown that lin28 knockdown inhibits the ability of pluripotent cells from the embryo to differentiate into mesoderm in response to growth factor signals and that the expression levels of genes activated by these signals are reduced in lin28 morphant embryos *in vivo*, when germ layer specification is occurring. We next investigated whether lin28 knockdown interferes with ERK/MAP kinase phosphorylation, which is a characteristic, direct response to FGF signalling (Umbhauer et al., 1995; Christen and Slack, 1999; Branney et al., 2009). Fig. 4E is a western blot showing that activin or FGF treatment of animal cap explants upregulates the levels of ERK phosphorylation at early gastrula stages. However, ERK phosphorylation in response to FGF is reduced following lin28 knockdown, indicating that lin28 function is involved in the very earliest responses of pluripotent cells to FGF signalling.

Activin treatment of animal cap explants also induces ERK phosphorylation. Strikingly, lin28 knockdown greatly reduces ERK phosphorylation in response to activin. In contrast to ERK activation by FGF signalling, ERK phosphorylation in response to activin is a secondary, indirect response, requiring the downstream activation of transcription from FGF ligand-encoding genes (Schulte-Merker and Smith, 1995). As mentioned above, a pathway that involves primary mesoderm inducers, such as nodal/activin, activating the transcription of FGFs, including *FGF4* and *FGF8* as secondary mesoderm inducers/maintenance factors, is believed to operate during mesoderm formation *in vivo*. We investigated whether lin28 function is necessary for the normal activation of transcription of the endogenous *FGF4* and *FGF8* genes *in vivo*. The expression levels of *FGF4* and *FGF8* are significantly reduced in lin28 morphant embryos at early gastrula stages (Fig. 4F).

Heterochronic effects of lin28 inhibition during early amphibian development

lin-28 was originally identified as a heterochronic gene in *C. elegans*. *lin-28* mutations lead to the aberrant, precocious activation of later developmental events (Ambros and Horvitz, 1984; Moss et al., 1997). We examined whether the inhibition of lin28 function in *Xenopus* also leads to the misregulation of developmental events in

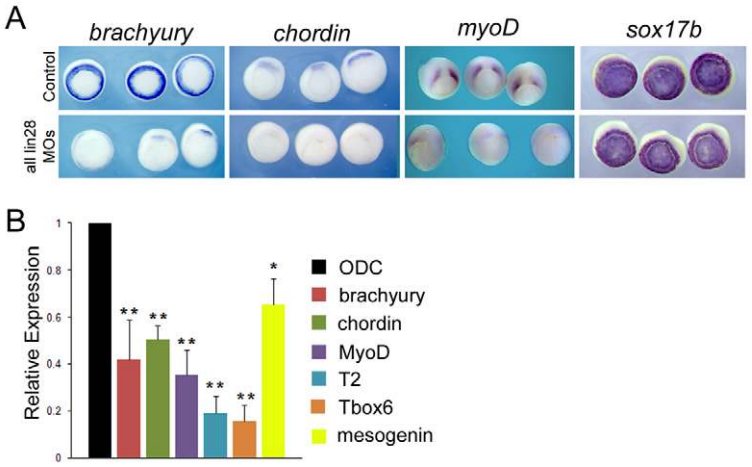

Fig. 3. Effect of lin28 knockdown on mesodermal gene expression. (**A**) Expression of *brachyury*, *chordin*, *myoD* and *sox17b* in control and lin28 morphant *X. tropicalis* embryos assessed by *in situ* hybridisation at gastrula stage 10.5. (**B**) Expression of the indicated mesodermal markers in lin28 morphants relative to expression in control embryos as assessed by Q-PCR at stage 10.5. **P*<0.05, ***P*<0.005 (Student's *t*-test), for morphant expression compared with control expression. *ODC* is a ubiquitously expressed housekeeping gene. Error bars indicate s.d.

the early amphibian embryo. To this end, we investigated the timing of activation of gene transcription and growth factor signalling in the early embryo during the process of germ layer specification.

Fig. 5 represents a developmental timecourse of gene expression in synchronously developing, sibling control and lin28 morphant embryos cultured at 27°C, with time points set at 30-minute intervals. This timecourse encompasses the pre-MBT to early gastrula stage of development, with the latter time points corresponding to the stages at which our analyses detect aberrant mesodermal gene expression in lin28 morphants. Transcription from *nodal-related 5* (*nr5*) and *nr6* is upregulated rapidly in control embryos following the MBT at mid-blastula stage 8. The upregulation of *nr1* expression in normal development is a slightly delayed response, relative to *nr5* and *nr6*. At the time resolution provided by our experiments, we see no evidence of precocious *nr5* or *nr6* transcription in response to lin28 knockdown; however, the expression of these genes at stage 8 is significantly higher in morphants (Fig. 5B,C). Subsequently, the shapes of the expression profiles from control and morphant embryos for *nr5* and *nr6* are very similar; however, the profiles in morphants are shifted to the

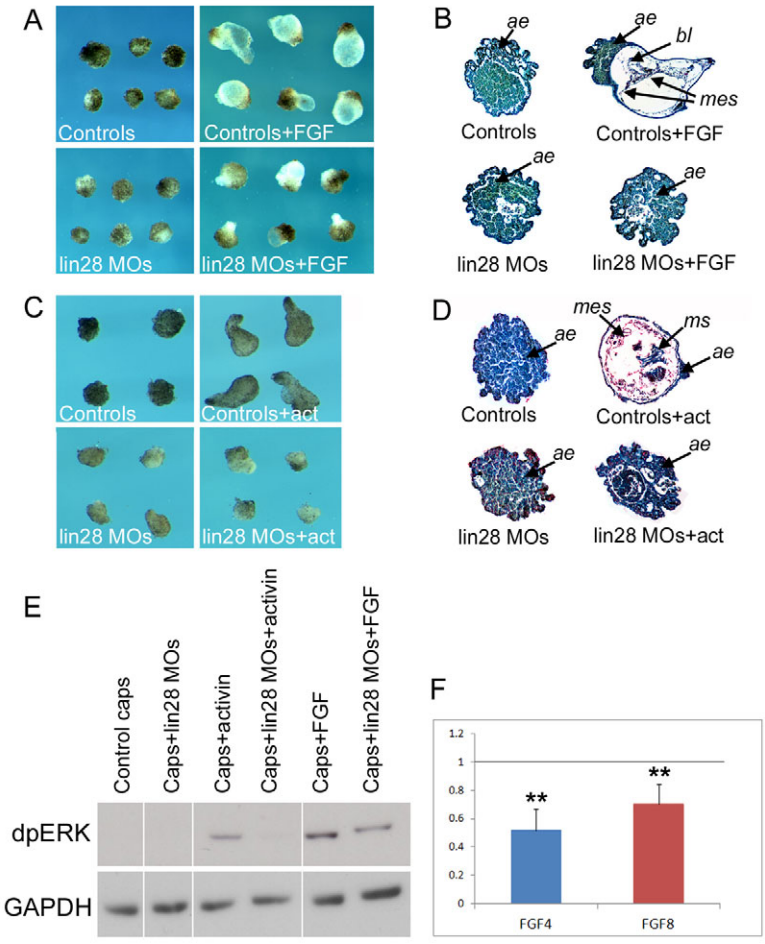

Fig. 4. Effect of lin28 knockdown on FGF and activin/nodal signalling. (**A**,**C**) Animal cap explants from control and lin28 morphant *X. tropicalis* embryos, with or without FGF (A) or activin (C) treatment. (**B**,**D**) Histology on sections of animal caps from A and C, respectively. *ae*, atypical epidermis; *bl*, blood; *mes*, mesenchyme; *ms*, muscle. (**E**) Western blots showing dpERK expression levels in animal cap explants from control and lin28 morphant embryos, with or without activin or FGF treatment. GAPDH, loading control. (**F**) Expression levels of *FGF4* and *FGF8* in lin28 morphants at stage 10.5 relative to controls as assessed by Q-PCR. Solid line represents mRNA levels in corresponding control embryos (set at 1). ***P*<0.005 (Student's *t*-test), for morphant expression compared with control expression. Error bars indicate s.d.

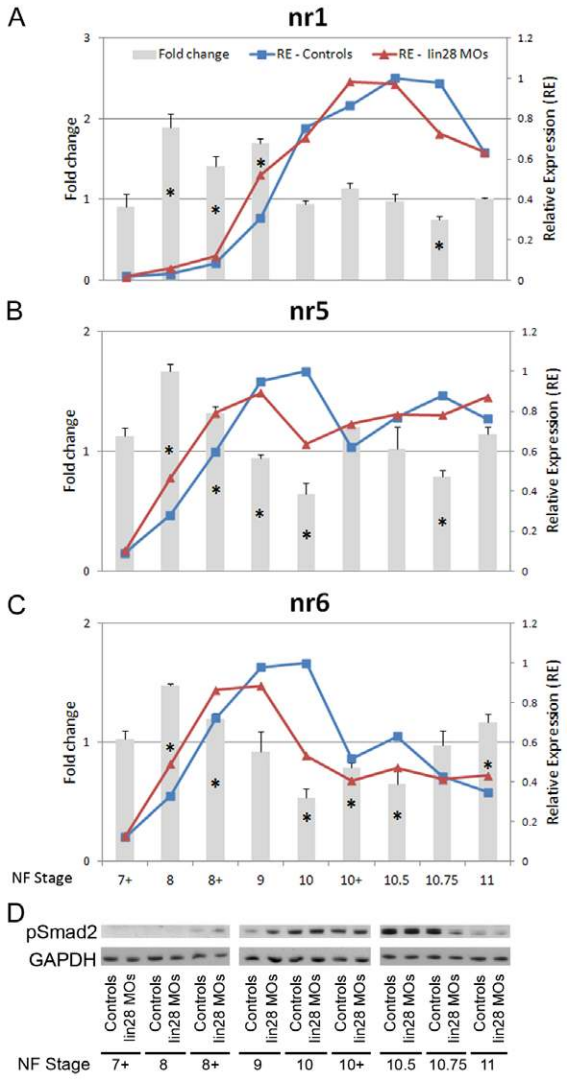

Fig. 5. Effects on the temporal regulation of nodal-related pathway components in lin28 morphants. (**A-C**) Temporal expression profiles of *nr1* (A), *nr5* (B) and *nr6* (C) from NF blastula stage 7+ to gastrula stage 11 as assessed by Q-PCR in control and lin28 morphant *X. tropicalis* embryos. Relative expression levels were calculated as the proportion of the maximum expression level in controls (set at 1) and fold change values are expressed relative to controls (set at 1). **P*<0.05 (Student's *t*-test), for morphant compared with control expression. (**D**) Western blot showing the expression levels of p-Smad2 at the NF stages analysed above. GAPDH, loading control. Error bars indicate s.d.

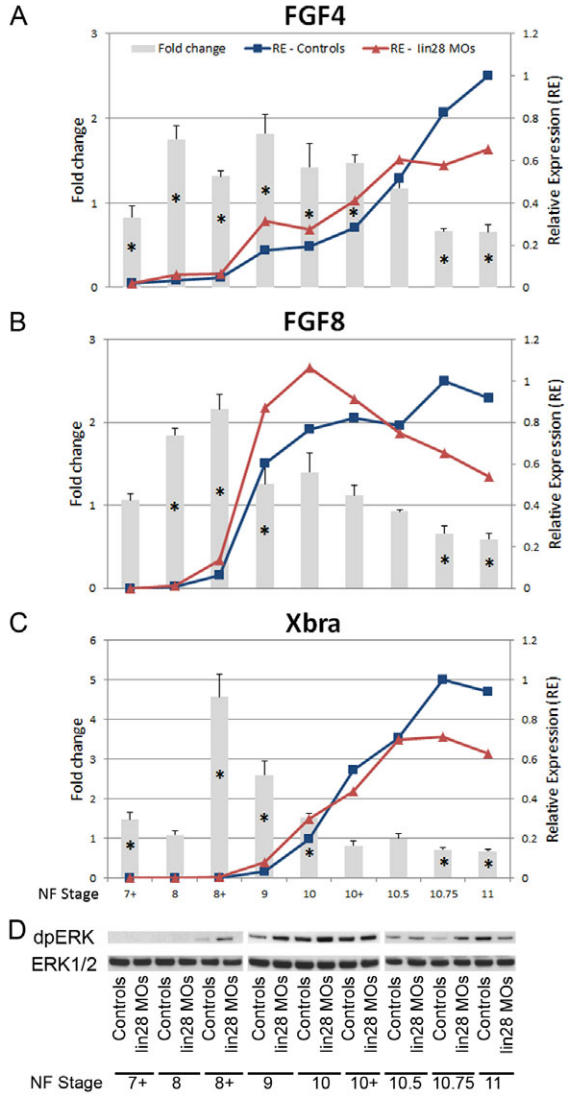

Fig. 6. Effects on the temporal regulation of FGF pathway components in lin28 morphants. (**A-C**) Temporal expression profiles of *FGF4* (A), *FGF8* (B) and *Xbra* (C) from NF blastula stage 7+ to gastrula stage 11 as assessed by Q-PCR in control and lin28 morphant *X. tropicalis* embryos. Relative expression levels and fold change values were calculated as in Fig. 5. **P*<0.05 (Student's *t*-test), for morphant compared with control expression. (**D**) Western blot showing the expression levels of dpERK at the NF stages analysed above. GAPDH, loading control. Error bars indicate s.d.

left. Thus, peak levels of *nr5* and *nr6* are reached earlier and the dip in expression levels, which is detected at the start of gastrulation in controls, occurs somewhat earlier in lin28 morphants. A similar shifted expression profile is also seen with *nr1*, with initial expression levels higher in morphants and the peak expression level being achieved earlier (Fig. 5A).

Smad2 is a key intracellular effector that is rapidly phosphorylated and activated in response to activin/nodal signalling (Baker and Harland, 1996; Bourillot et al., 2002; ten Dijke and Hill, 2004). The developmental timecourse of phospho-Smad2 (p-Smad2) levels was analysed and, in keeping with the expression profiles of the nodal-related genes, the initial level of p-Smad2 is higher and the reduction of p-Smad2 levels at early to mid-gastrula stages occurs somewhat earlier in morphants than in controls.

A similar timecourse analysis was also undertaken with the FGF ligand genes *FGF4* and *FGF8*, and with *Xbra*, which is activated as an immediate early response to both FGF and activin signalling. Again, the initial expression of all three genes is significantly elevated in morphants relative to control siblings (Fig. 6A-C), as are levels of activated ERK (Fig. 6D). However, in keeping with our previous results we find that gene expression levels are significantly reduced by early to mid-gastrula stages in lin28 morphant embryos.

let-7 miRNAs in *Xenopus* development

Recent evidence suggests that Lin28 proteins regulate the pluripotent state in stem cells *in vitro* through the negative regulation of let-7 miRNA biogenesis (Viswanathan et al., 2008;

Hagan et al., 2009; West et al., 2009). We examined whether the observed developmental effects in lin28 morphants correlate with altered let-7 expression levels. Fig. 7A shows the expression profiles in early *Xenopus* development of the mature forms of let-7a, let-7f and let-7g, which are regulated by Lin28 in mammalian cell culture (Viswanathan et al., 2008; Heo et al., 2009). We note that cycle threshold values (Cts) for all three let-7 miRNAs are relatively high, indicating that these miRNAs are present at low levels during early *Xenopus* development (data not shown).

Lin28 binding to the terminal loop region of immature let-7 miRNAs is crucial for regulating biogenesis of the mature miRNAs (Piskounova et al., 2008; Heo et al., 2009; Lightfoot et al., 2011). Cell lysates from *Xenopus* embryos overexpressing *lin28a1*, *lin28a2* or *lin28b* mRNAs contain activities that are able to bind to the terminal loop of let-7g (Fig. 7B-D). Moreover, we confirm that the binding activities correspond to the overexpressed lin28 proteins by showing that the addition of a relevant anti-lin28 antibody is able to supershift each riboprotein complex.

These data indicate that let-7 miRNAs are potential regulatory targets of lin28 proteins during amphibian development and it is possible that elevated levels of mature let-7 miRNAs contribute to the phenotype arising from lin28 knockdown. Indeed, overexpression of mature let-7a leads to a phenotype characterised by ventral bending of the main body axis, whereas overexpression of a mutant let-7 has less of an effect on axial development (supplementary material Fig. S4A,B). However, the phenotypic effects of let-7 overexpression are different from those produced by lin28 knockdown (compare with Fig. 2A).

We next analysed the levels of mature let-7a, let-7f and let-7g miRNAs in lin28 compound knockdown embryos (Fig. 7E). Surprisingly, we found no significant changes in the overall levels of let-7 miRNAs in lin28 morphant embryos at early gastrula stage 10.5. This is the stage when our analyses indicate that changes in mesodermal gene expression are already apparent in lin28 morphants. Therefore, the observed effects from lin28 knockdown on early mesoderm specification are unlikely to be mediated through a lin28/let-7 regulatory pathway. By contrast, by early tailbud stage 22, the overall level of let-7g is significantly higher in lin28 morphants compared with control embryos, indicating a role for lin28 proteins in regulating the biogenesis of at least some let-7 miRNAs during later development.

DISCUSSION

A model for lin28 function during germ layer specification

lin28 genes are expressed in pluripotent cells of the amphibian embryo. We propose that lin28 function is required in order for the pluripotent cells of the amphibian embryo to respond appropriately to mesoderm-inducing growth factors.

During amphibian development, both *lin28a* and *lin28b* are regulated by the FGF signalling pathway. In addition, knockdown of lin28 function inhibits the ability of pluripotent embryonic cells

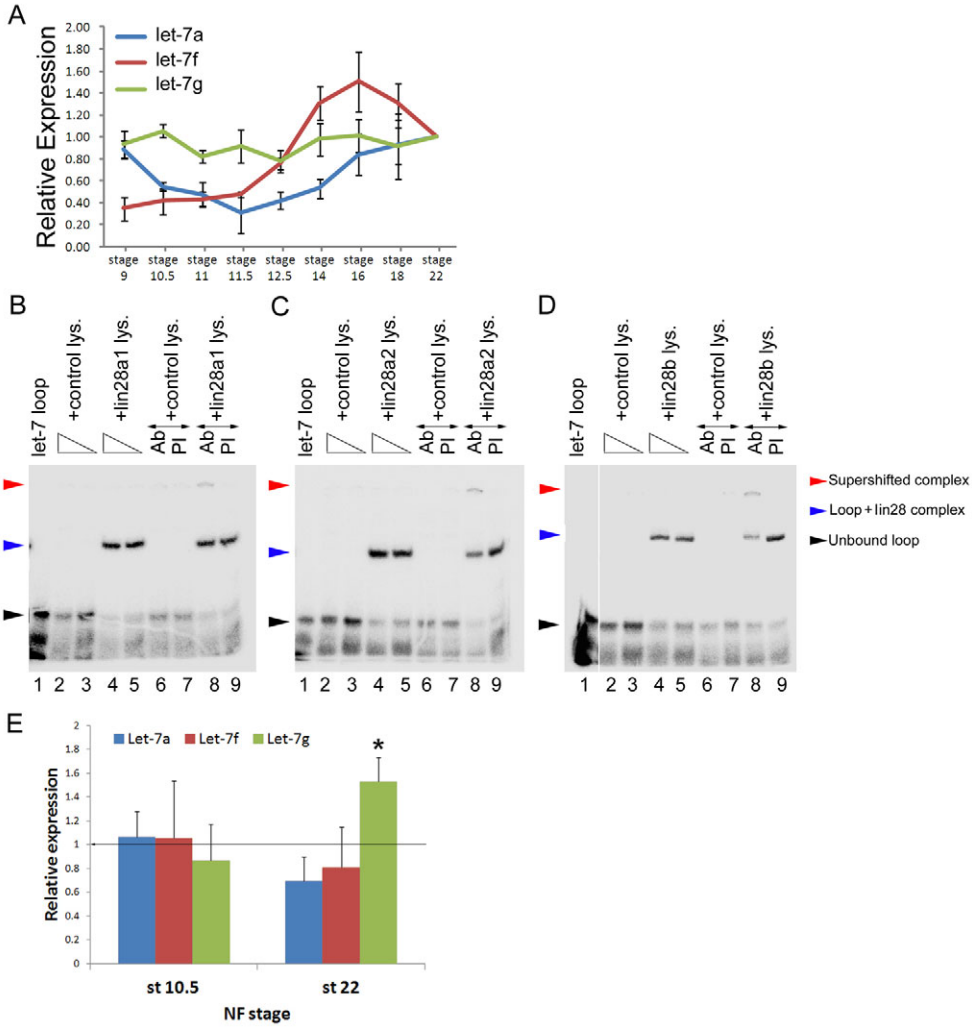

Fig. 7. let-7 in *Xenopus* development. (**A**) Q-PCR analysis of let-7a, let-7f and let-7g expression from stage 9 to stage 22. Expression is calibrated to stage 22 for each gene. (**B-D**) RNA EMSA supershifts showing let-7g terminal loop RNA-binding activity of dilutions of protein extracted from *Xenopus* embryos overexpressing lin28a1 (B), lin28a2 (C) or lin28b (D) proteins. Lane 1, labelled let-7g terminal loop alone; lanes 2 and 3, let-7g plus 1:4 and 1:8 dilutions of control embryo lysate, respectively; lanes 4 and 5, let-7g plus 1:4 and 1:8 dilutions, respectively, of lysate from lin28-overexpressing embryos; lane 6, let-7g plus 1:4 dilution of control embryo plus relevant lin28 antibody; lane 7, let-7g plus 1:4 dilution of control embryo lysate plus relevant pre-immune serum; lane 8, let-7g plus 1:4 dilution of lin28-overexpressing embryo lysate plus relevant lin28 antibody; lanes 9, let-7g plus 1:4 dilution of lin28-overexpressing embryo lysate plus relevant pre-immune serum. (**E**) Q-PCR analysis of relative expression of let-7 miRNAs in lin28 morphants compared with control embryos at stage 10.5 and 22. Solid line represents miRNA levels in corresponding control embryos (set at 1). **P*<0.05 (Student's *t*-test), for morphant compared with control expression. Error bars indicate s.d.

to differentiate into mesoderm in response to FGF signals. Knockdown of lin28 function also compromises the ability of embryonic cells to respond to the mesoderm-inducing activity of activin, a TGFβ superfamily member that mimics the activity of endogenous mesoderm-inducing factors, such as nodal-related proteins. Furthermore, lin28 function is required for the appropriate early expression of FGF and nodal-related genes. Taken together, our data support a model in which *lin28* genes are components of the regulatory pathway involved in the specification and patterning of the amphibian mesoderm, modulating responses both upstream and downstream of FGF signalling.

Transcriptional regulation of *lin28* genes

Current understanding of the transcriptional regulation of vertebrate *Lin28* genes is limited. Our data suggest a role for FGF signalling in regulating the expression of *Xenopus lin28* genes. However, unlike some other known transcriptional targets of FGF signalling in amphibian development, such as *brachyury*, inhibiting FGF signalling does not completely block the expression of *Xenopus lin28a* or *lin28b* (Amaya et al., 1993). This suggests that additional pathways are likely to contribute to the transcriptional regulation of *lin28* genes in the amphibian embryo. We note that during the preparation of this manuscript it was reported that expression of *Lin28b* is regulated by FGF in the developing chick embryo (Bobbs et al., 2012), suggesting that an FGF-Lin28 regulatory axis might be a conserved feature of vertebrate embryo development.

Lin28 regulation of timing in early amphibian development

Lin28 knockdown leads to precocious elevation and subsequent precocious reduction in signalling by both the FGF and nodal pathways, indicating that lin28 function is involved in regulating the timing of signalling events in the early amphibian embryo. Recent evidence suggests that amphibian ventx transcription factors, which have Nanog-like activity, are also involved in regulating the timing of cell commitment to specific lineages in *Xenopus* (Scerbo et al., 2012). Both Lin28 and Nanog-related factors are implicated in the maintenance of the pluripotent state in mammalian stem cells. We propose that, similarly to ventx, lin28 participates in regulating the timing of responses to signalling pathways involved in cell lineage restriction in early amphibian embryogenesis.

lin-28 was originally identified as a heterochronic gene that encodes a factor promoting early developmental fates in *C. elegans* (Ambros and Horvitz, 1984; Moss et al., 1997). Subsequent evidence indicating that Lin28 proteins are involved in regulating the pluripotent state of mammalian stem cells in culture has further supported their role as factors promoting early stem cell phenotypes versus later differentiated cell fates (reviewed by Viswanathan and Daley, 2010). Our data provide the first evidence that *Lin28* genes are involved in regulating the timing of events in the early development of a vertebrate embryo, as opposed to stem cells in culture.

However, the role of lin28 in mesoderm development appears to be complex; although lin28 knockdown leads to precocious elevation of pathways involved in mesoderm specification, subsequent patterning and differentiation of the mesoderm are impaired. Thus, amphibian *lin28* genes appear to function as developmental 'gatekeepers' involved in regulating the transition from the pluripotent to mesodermal cell fate. Evidence indicates that this might represent a conserved function of *Lin28* genes, as it has recently been shown that Lin28a knockdown inhibits mesodermal gene expression during murine ES cell differentiation (Wang et al., 2012).

The proposed gatekeeper role of Lin28 proteins provides an intriguing link with the recently demonstrated function of FGFs as regulators of the transition from the pluripotent state to lineage restriction in ES cells. FGF signalling is required to allow ES cells to respond appropriately to signals involved in specifying particular lineages, including the mesoderm (Kunath et al., 2007). It will be interesting to determine whether an FGF-Lin28 axis, similar to that proposed in this study, operates in mammalian ES cells in culture.

let-7-independent function of lin28

Much recent work has focussed on Lin28 proteins as negative regulators of let-7 miRNA biogenesis. Although this is undoubtedly a key feature of Lin28 function, in some situations there is increasing evidence that Lin28 proteins have other, let-7-independent functions. A recent study indicates that LIN28A can function independently of regulating let-7 abundance during the differentiation of P19 embryonal carcinoma cells (Balzer et al., 2010). Furthermore, during *C. elegans* development LIN-28 has been shown to act through a two-step mechanism involving an initial let-7-independent phase and subsequent let-7-dependent phase (Vadla et al., 2012).

Our data also suggest early let-7-independent and later let-7-dependent roles for lin28 during amphibian development. It has been proposed that the initial let-7-independent function in *C. elegans* involves LIN-28 directly interacting with, and promoting translation from, target mRNAs (Vadla et al., 2012). There is increasing evidence that such direct, miRNA-independent regulation of mRNA translation by Lin28 proteins is a key element of their function, and a wide range of mRNA targets have been identified (Polesskaya et al., 2007; Xu and Huang, 2009; Qiu et al., 2010; Jin et al., 2011; Peng et al., 2011; Lei et al., 2012). We speculate that lin28 function during early amphibian development might similarly involve direct regulation of mRNA translation. This would seem particularly relevant to lin28b, which is present before the onset of zygotic transcription and is therefore a potential regulator during a phase of development that is critically dependent upon the translational regulation of maternally deposited mRNAs. In this regard, we note that Y-box proteins, which like lin28 contain a cold shock domain, are involved in translational control during the maternal phase of *Xenopus* development (Bouvet and Wolffe, 1994; Matsumoto and Wolffe, 1998). At present, the target mRNAs with which lin28 proteins might interact during early amphibian development remain to be identified.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material available online at
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