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Transient modification of *lin28b* expression - Permanent effects on zebrafish growth

Jaakko T. Leinonen[a], Yu-Chia Chen[b], Taru Tukiainen[a], Pertti Panula[b], Elisabeth Widén[a,*]

[a] Institute for Molecular Medicine Finland (FIMM), University of Helsinki, P.O. Box 20 (Tukholmankatu 8), Helsinki, 00014, Finland
[b] Department of Anatomy and Neuroscience Center, University of Helsinki, P.O. Box 63, (Haartmaninkatu 8), Helsinki, 00014, Finland

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ABSTRACT

Recent genome-wide association studies and mouse models have identified *LIN28B* as a gene affecting several pubertal timing-related traits and vertebrate growth. However, the exact biological mechanisms underlying the associations remain unknown. We have explored the mechanisms linking *LIN28B* with growth regulation by combining human gene expression data with functional models. Specifically, we show that 1) pubertal timing-associated genetic variation correlates with *LIN28B* expression in the pituitary and hypothalamus, 2) down-regulating *lin28b* in zebrafish embryos associates with aberrant development of *kiss2*-neurons, and 3) increasing *lin28b* expression transiently by synthetic mRNA injections during embryogenesis results in sustained enhancement of zebrafish growth. Unexpectedly, the mRNA injections resulted in advanced sexual maturation of female fish, suggesting that *lin28b* may influence pubertal timing through multiple developmental mechanisms. Overall, these results provide novel insight into *LIN28B* function in vertebrate growth regulation, emphasizing the importance of the gene and related genetic pathways for embryonic and juvenile development.

1. Introduction

Age at pubertal onset varies widely within the general population and several monogenic syndromes are known to cause clinically significant changes in the timing of puberty (Gajdos et al., 2010; Herbison, 2007). Typically, mutations associated with these syndromes affect genes encoding for hormone receptors that bind crucial hormones regulating vertebrate growth, e.g. the gonadotropin-releasing hormone (*GnRH)* and kisspeptin *(KISS1)* genes (Gajdos et al., 2010; Herbison, 2007; de Roux et al., 1997, 2003). More recently, Genome-Wide Association Studies (GWAS) have identified hundreds of loci in our genomes where common genetic variation affects the timing of puberty in more subtle ways (Elks et al., 2010; Perry et al., 2014; Day et al., 2017). Some of these loci implicate genes previously associated with GnRH-secretion, pituitary development and function, but for many others, including the locus close to *LIN28B*, the exact biology behind the association signal, remains mostly elusive (Elks et al., 2010; Perry et al., 2014; Ong et al., 2009).

Interestingly, variants in the *LIN28B* locus associating with pubertal timing also associate with variation in traits such as height, finger length ratio and adiposity, all of which are features connected to hormonal regulation during development (Lettre et al., 2008; Cousminer et al., 2013; Medland et al., 2010; Leinonen et al., 2012; Widén et al., 2010). Additionally, data from model organisms ranging from nematodes to mice have robustly shown the *LIN28* genes to be regulators of developmental timing and vertebrate growth (Ambros and Horvitz, 1984; Zhu et al., 2010). Published studies on mice, frog and zebrafish suggest that permanent knockout or knockdown of *LIN28B* homologues during early embryogenesis may have detrimental effects on further development, with phenotypes ranging from lethality or growth defects to differences in pubertal timing and glucose intolerance (Zhu et al., 2010, 2011; Ouchi et al., 2014; Shinoda et al., 2013a; Corre et al., 2016; Faas et al., 2013). Some studies and animal models further implicate sex-specific regulation of growth and puberty by *LIN28* genes and their antagonists' (*let7*-miRNAs). Interestingly, several studies have established the expression of these genes in the hypothalamic-pituitary-gonadal-axis (HPG-axis), and shown how they contribute to cancer, neurogenesis, and RNA regulation (Leinonen et al., 2012; Widén et al., 2010; Zhu et al., 2010; Corre et al., 2016; Shinoda et al., 2013b; Gaytan et al., 2013; Grieco et al., 2013; Sangiao-Alvarellos et al., 2013, 2015; Balzer et al., 2010; Viswanathan et al., 2008; Molenaar et al., 2012; Piskounova et al., 2011; Wilbert et al., 2012). Nonetheless, the precise mechanisms associating *LIN28B* with variation in growth and development in human population studies remain unclear. In particular, it remains unknown whether these mechanisms might be limited to specific cell types, tissues and periods of development. With our study, we

* Corresponding author. Institute for Molecular Medicine Finland (FIMM), University of Helsinki, P.O. Box 20, FI-00014, Finland.
E-mail address: elisabeth.widen@helsinki.fi (E. Widén).

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especially wanted to determine how the actions of *lin28b* during embryogenesis might contribute to the GWAS-associated phenotypes.

This study combines knowledge from the Genotype-Tissue Expression (GTEx) database (The GTEx Consortium, 2015), providing high resolution gene expression data across dozens of human tissue types, with zebrafish models assessing the consequences of transient *lin28b* dysregulation during embryogenesis. The results highlight the importance of controlled *lin28b* expression during fetal development. Specifically, our data implies that in humans the pubertal timing-associated SNPs in the *LIN28B* locus tag changes in *LIN28B* expression in key tissues related to puberty, the pituitary and the hypothalamus. Moreover, our data indicate that *lin28b* has the potential to contribute to the development and function of the hypothalamus, and that even transient *lin28b* overexpression during embryogenesis is enough to alter fish growth trajectories.

2. Materials and methods

2.1. Human gene expression data from GTEx

The Genotype-Tissue Expression (GTEx) Project facilitates the study of human gene expression and regulation and its relationship to genetic variation by providing the scientific community with a resource of rich gene expression and genotype data from hundreds of individuals and thousands of samples from a diverse set of human tissues (The GTEx Consortium, 2015). We queried this database to understand the breadth and tissue-specificity of expression and the genetic regulation of the genes in the *LIN28B* locus.

For the current analysis release (GTEx Analysis V6 release, dbGaP Accession phs000424.v6.p1), the GTEx project has collected tissue samples from 554 postmortem donors (187 females, 357 males; age range 20–70), produced RNA sequencing data from 8555 tissue samples spanning 54 unique tissue types and generated genotype data for up to 449 donors. The data production and analysis procedures are available through the GTEx Portal (http://gtexportal.org) and described in detail in (The GTEx Consortium, 2015).

The GTEx eQTL results for pubertal timing and longitudinal growth associated SNPs rs7759938 and gene expression data [per sample gene Reads Per Kilobase Million (RPKM) values] for *LIN28B* and other genes in the locus used in this study were downloaded from the GTEx Portal.

2.2. Fish

Zebrafish were obtained from the breeding line that has been maintained in the Panula laboratory for more than 10 years (Kaslin and Panula, 2001; Kaslin et al., 2004). Developing embryos were staged in hours post-fertilization (hpf) or days post-fertilization (dpf) as described previously (Kimmel et al., 1995). Embryos and larvae were raised at 28 °C. For *in situ* hybridization, the embryos were dechorionated manually and treated with 0.03% phenylthiourea added in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.4 mM $CaCl_2$, and 0.16 mM $MgSO_4$) to inhibit pigmentation. For dissection of the 120-day old fish brain the fish were anesthesized with 0.03% tricaine. The experiments including measuring fish size were performed with the researcher taking measures blinded for the fish group information. Puberty and sex were assessed by examining the growth of gonads following the guidelines from (Chen and Ge, 2013), with egg production determined as fish having eggs that were immediately apparent after dissecting female fish. For experiments comparing fish size during juvenile growth the number of fish per tank (tank size = 3 L) was kept similar between the groups (ranging between 7 vs. 7 fish to 20 vs. 21 fish). In our growth experiments we did not track the growth of individual fish but each fish was measured only once at a single timepoint. The permits for the experiments were obtained from the Office of the Regional Government of Southern Finland in agreement with the ethical guidelines of the European convention for the protection of vertebrate animals used for experimental and other scientific purposes.

2.3. Morpholino oligonucletide/synthetic mRNA design and preparation

Antisense morpholino oligonucleotides (MO) were designed to target the initiator codon and a splice donor site between exon 1 and exon 2 of *lin28b* (*lin28b* MO1, 5′ GCGCGGCCCTCCTTCGGCCATGTTT′3; *lin2*8b MO, 5′CCCTCCTGTGCGGTCAAGAGAGAAA′3. These and a *p53*-blocking MO and a standard control MO (ctrl) (*p53* 5′GCGCCATTGCT TTGCAAGAATTG′3, ctrl 5′CCTCTTACCTCAGTTACAATTTATA′3) were obtained from Gene Tools LLC, Philomath, OR. The morpholinos were injected with concentrations ranging from 0.75 ng to 6 ng/embryo. For most of the experiments, including the qPCR studies, we used the splice-site blocking MO to knock down *lin28b* expression. To control for potential off-target effects associated with MO use, we assessed the dose response curves of several MO doses and refrained from using doses clearly resulting in lethality and gross developmental defects for the embryos. The inhibition of *lin28b* mRNA synthesis by MOs were confirmed by qPCR. mRNA rescue experiments co-injecting MO and mRNA were utilized to assess the specificity of the observed phenotypes.

lin28b full-length open reading frame cDNA constructs were prepared by RT-PCR using Phusion High-Fidelity PCR Master Mix (Thermo Fischer Scientific, Waltham, MA). The primers for *lin28b* cloning were F 5′GATTTCGCTGGAACTTTG′3 and R 5′CTGTTTGAGGTAGATGAT TTC′3, amplifying the whole predicted ORF of *lin28b* (213aa) + 126bp of 5′ and 58bp of 3′ UTR. PCR amplicons were cloned into pGEM-T Easy vector (Promega, Madison, WI) and verified by sequencing. The insert sequence was subcloned into the expression vector pMC kindly donated by Dr. Thomas Czerny. Plasmid was linearized with Thermo Fisher Scientific Fast Digest NotI for mRNA synthesis. Capped full-length open reading frame transcripts were generated by the mMESSAGE mMACHINE T7 kit (Ambion, Austin, TX). Synthetic mRNA was injected in concentrations ranging from 125 pg to 1 ng per embryo.

2.4. Whole-mount in situ hybridization (WISH)

WISH was performed on 4% paraformaldehyde (PFA)-fixed embryos and 7-dpf dissected larvae brains based on the protocol by Thisse & Thisse (Thisse and Thisse, 2008). Antisense and sense digoxigenin (DIG)-labeled RNA probes were generated using the DIG RNA labeling kit (Roche Diagnostics) following the instructions of the manufacturer. Prehybridization and hybridization were conducted at 63 °C for all riboprobes. *In situ* hybridization signals were detected with sheep anti-digoxigenin-AP Fab fragments (1:10,000, Roche Diagnostics). Color was developed with chromogen substrates NBT and BCIP (nitro blue-tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate).

2.5. RNA isolation, cDNA synthesis and Quantitative real-time PCR (qPCR)

Total RNA was extracted using miRNeasy mini Kit (Qiagen Inc., Valencia, CA) according to the instructions of the manufacturer, from 20 to 25 pooled embryos/larvae collected at 1, 2, 3 and 5-dpf or from a single 120d old fish brain. 0.5–1.5 μg of RNA was reverse-transcribed using SuperScript® VILO cDNA Synthesis Kit. qPCR was performed with the Light Cycler® 480 (F. Hoffmann-La Roche Ltd, Switzerland) instrument using iQ™ SYBR® green Supermix (Bio-Rad, Hercules, CA) as instructed. 0.5 μl of 5 mM primers and 1 μl of 1:14 dilution of the cDNA was used per 10 μl reaction. Primers for amplification were designed with Sigma OligoArchitect™ and Primer-BLAST (NCBI). Primer sequences and cycling conditions are shown in Table A.1. Fluorescence changes were monitored after every cycle. Dissociation curve analysis was performed to ensure only a single amplicon was obtained. Results were obtained with the LightCycler® 480 software and after quality control, data were normalized and calculated based on Ct values. Reactions were set up as duplicates and relative expression levels were calculated based on two reference genes (*b-actin* and *eif-1a*), using the

2-δδCT method.

2.6. Imaging

Bright-field images of WISH embryos were obtained with Leica DM IRB inverted microscope and a DFC 480 change-coupled device camera using 5x, 10x and 20x magnification. Pictures of live 3-6d fish drugged with 0.02% tricaine were taken with Leica DMi1 and the fish were measured from the images using the Leica Application Suite 4.5 software. The 2dpf fish in Fig. A2 were imaged with NIKON Coolpix E4500, integrated to Leica MZFLIII.

2.7. Statistical analysis

Data analysis was performed in R version 3.0.2. qPCR results for 1-5d fish, showing unequal variance between groups, were analyzed with Welch ANOVA combined with a Games-Howell post-hoc test. qPCR results for 120d fish brain RNA expression, as well as fish length and head width at 6 days were analyzed with one-way ANOVA combined with post-hoc Tukey HSD. Growth data comparing WT and mRNA injected fish, following normal distribution were assessed by a logistic regression model adjusting for fish age. Based on our own observations and data from Chen and Ge (Chen and Ge, 2013), we assumed the fish growth to be approximately linear between 60d and 120d. To evaluate the effect on growth in more detail, we further performed Welch two sample t-tests for each time point and sex separately. To assess sex-specificity of the growth results we fitted sex into the model as an additional covariate. For a comparison of sex/egg ratios in adult fish, and phenotype ratios in embryos, we utilized Fisher's exact test, using Bonferroni correction for multiple testing when appropriate. For 1-5d qPCR data, a set of 20–25 embryos collected either at 1,2,3 or 5d represents a single experimental unit. In the 120d qPCR experiments, pooled results from 7 to 8 fish brain represent a single experimental unit. In growth experiments, either a well containing a single 6d larva, or a tank containing 7 to 21 juvenile fish (injected fish grown in separate tanks from the controls), was considered as an experimental unit. All the fish used for statistical calculations in each well/tank were measured only at a single time point.

3. Results

3.1. Pubertal timing associated SNPs associate with LIN28B expression in the pituitary and hypothalamus

GTEx contains genotypes and RNA expression data from post-mortem human tissues, thus providing a resource to study how genetic variation affects tissue-specific RNA expression. For this study we explored the database, first concentrating on the level of *LIN28B* expression across 53 adult tissues. Although the level of expression in most tissues was generally very low, some subregions of the brain showed relatively higher expression levels for *LIN28B*. Interestingly, out of all the examined brain regions (N = 14) hypothalamus and pituitary appeared to have the highest expression levels for *LIN28B*. Among all tissues the expression peaked in the testis (Fig. 1).

Previously, mouse models have suggested that increased *LIN28* expression leads to delayed puberty, and according to a recent report increased *LIN28B* expression in the pituitary associates with later AAM also in humans (Day et al., 2017). Since the hypothalamus, pituitary, ovaries, and testis all secrete hormones guiding puberty, *LIN28B* expression in these tissues might be especially relevant for the regulation of pubertal timing. To explore this possibility in more detail, we examined whether genetic variants in the *LIN28B* locus that have been previously associated with pubertal timing, including rs7759938, correlated with *LIN28B* expression in these tissues. We found that both in the hypothalamus and the pituitary *LIN28B* RNA expression varied significantly based on the rs7759938 genotype, ($P = 0.0057$ for the hypothalamus and 4.16e-05 for the pituitary), linking the pubertal timing associated haplotype with changes in *LIN28B* RNA expression levels in humans (Fig. 1). Nonetheless, in the testis, showing the highest average *LIN28B* expression, the same variants were not related to the gene's expression levels ($P = 0.21$).

Because GWAS-signals identify association to chromosomal regions typically spanning hundreds of kilobases, these generally pinpoint genetic loci containing multiple genes. Therefore, to verify that the effect on pubertal timing results from altered *LIN28B* expression, we evaluated whether the pubertal timing SNPs might affect the expression of other genes in the locus. Indicating that the pubertal timing associated SNPs primarily affect *LIN28B* expression in normal adult tissues, rs7759938 was not found to act as an eQTL for the other protein coding genes in the locus (*HACE1, BVES, POPCD3*, Fig. A1).

3.2. Expression of lin28b during embryogenesis

Although the GTEx data indicates that the common genetic variation in the *LIN28B* locus has the potential to change the expression levels of the gene in adult tissues, the *LIN28* genes generally exhibit prominent expression patterns during the early development, becoming downregulated later (Ouchi et al., 2014; Yang and Moss, 2003; Yokoyama et al., 2008). In vertebrates, the hypothalamus and pituitary start to develop already during embryogenesis. Therefore, using zebrafish as a model, we next characterized whether *LIN28B* might contribute to the formation of these tissues already during this period. We began by assessing the *lin28b* expression pattern in zebrafish embryos by *in situ* hybridization and qPCR, and, similarly to Ouchi et al. (2014) detected distinct and rapidly changing patterns of *lin2*8b expression over the first 24 h of development (Fig. 2A,B&C). Whilst the previously published *in situ* data concentrates on the first 36 hrs of development (Ouchi et al., 2014), we continued assessing *lin2*8b expression beyond this stage. In line with the established function of *lin28b* - linking expression of the gene with undifferentiated cells - we observed strong *lin28b* expression in developing organ primordia such as the fin buds still at 2d. Moreover, we detected staining in the midbrain-hindbrain boundary, a region with active neurogenesis (Fig. 2B&D) in both 2d and 3d embryos. However, by 7 days post fertilization, *lin28b* expression in the zebrafish brain was restricted to a specific network of neurons. Remarkably, these included neuronal projections to the hypothalamic-pituitary region (Fig. 2C).

A, B) *lin28b* expression in WT embryos at 1dpf (A) and 2dpf (B). The embryos show *lin28b* expression restricted to specific organ primordia such as the fin bud (FB, arrowhead) and NPZ = neuronal proliferation zone (arrow). C) 7d-WT larvae brain labeled with *lin28b* anti-sense probe. The brain contains a defined network of *lin28b* expressing cells, including projections to hypothalamus (Hy, arrow). D & E, comparisons of *lin28b* expression in 3d mRNA and MO injected embryos, showing strong and weak expression in the midbrain-hindbrain boundary at NPZ (arrows), and in the eyes (arrowheads). F & G, *gnrh3* expression at 3dpf in WT and MO embryos. Although some of the fish showed clearly smaller head and eye size, the *gnrh3* expression remained comparable in all fish. H & I, *kiss2* expression in WT and MO embryos. In line with the qPCR results, the MO injected fish showed a reduction in the intensity of *kiss2* staining. Scale bars = 100 μm. J) Changes in RNA expression for *lin28b*, *notch1a*, *gnrh3* and *kiss2* in *lin28b* MO or synthetic mRNA injected vs. WT and Rescue fish (combined injection of *lin28b* MO and mRNA) during the first five days of development. Over the five-day period, the MO injected embryos showed reduced and mRNA injected embryos increased expression of *lin28b* compared to WT ($P = 0.00039$ and $P = 0.0042$). No significant differences were observed in the expression of *notch1a* and *gnrh3*, however *kiss2* mRNA levels were significantly reduced in the MO group ($P = 0.033$, Welch ANOVA with Games-Howell post-hoc test, error bars = SEM). N = 12–20. K) Representative images of 3-day old zebrafish, including fish injected with *lin28b*-blocking morpholino and synthetic *lin28b* mRNA. In all

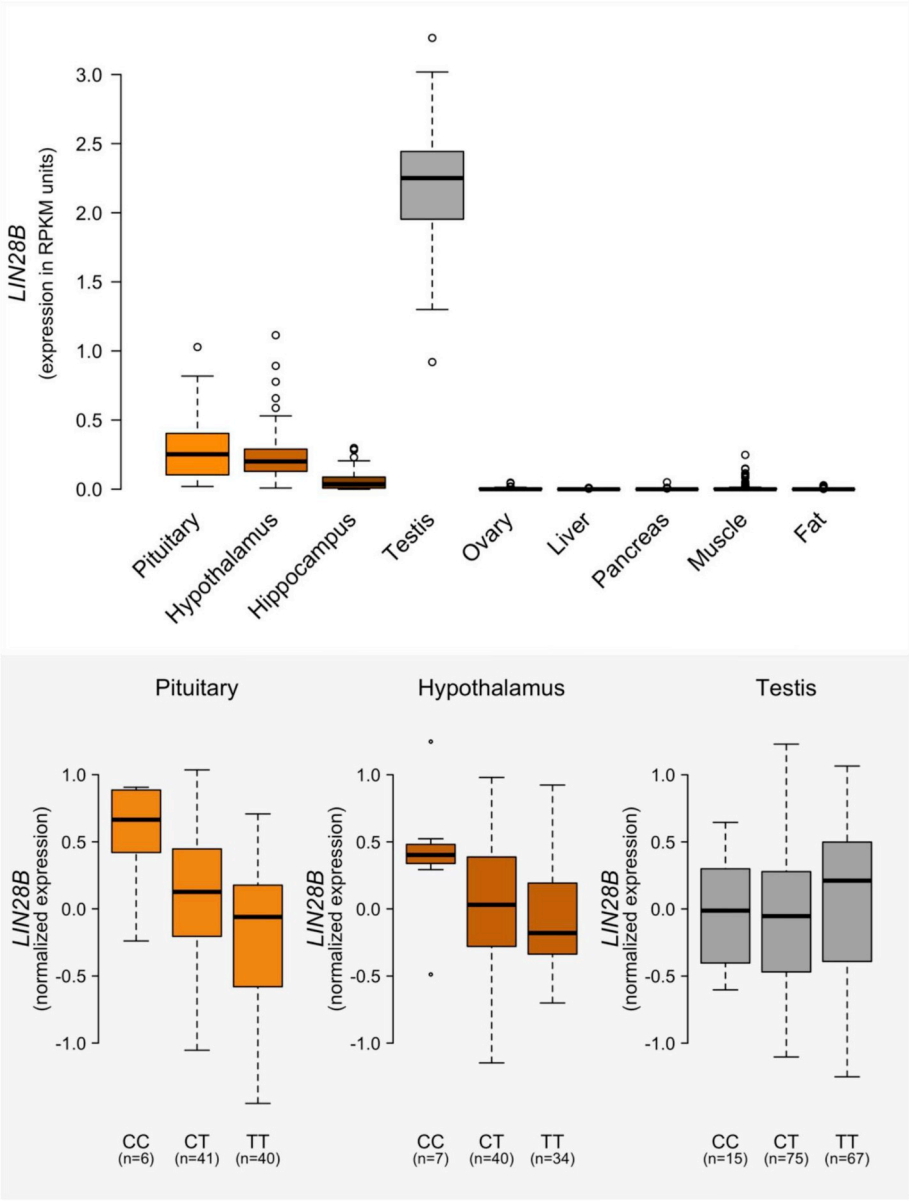

Fig. 1. *LIN28B* profiling in the GTEx database In adult humans, *LIN28B* is expressed at low levels across all tissues with the peak expression in the testis, pituitary and hypothalamus (upper panel). The pubertal timing associated SNP rs7759938 as an eQTL in the pituitary and hypothalamus, (P = 0.0057 for hypothalamus and 4.16e-05 for pituitary), but not in the testis (P = 0.21) (lower panel). The pubertal timing delaying allele (C) associates with increased *LIN28B* expression. The data shown as standardized mean expression, adjusted for principal components including sex and age.

injected groups, most fish receiving injections as embryos showed no gross phenotypic abnormalities. Control injections shown here included phenol red and H_20 only. Scale bars = 1 mm.

3.3. The effects of lin28b knockdown on zebrafish embryos

Having observed *lin28b* expression in tissues critically regulating pubertal timing and its prominent embryonic expression pattern, we sought to study the consequences of transient embryonic dysregulation of the gene. To alter gene expression levels, and to study any potential effects on brain development, we utilized anti-sense MO injections to knockdown the gene, synthetic mRNA injections to overexpress the gene, and combined injections of MO and mRNA to control for off-target effects of the MOs. We deliberately chose to work with injection doses that caused no gross morphological defects in the majority of the fish (Fig. 2K).

In previous studies, injection of zebrafish and *xenopus lavis* eggs with a large dose of *lin28b* blocking MO has caused embryonic lethality (Ouchi et al., 2014; Faas et al., 2013). Using high MO doses (6 ng and 3 ng), we similarly observed lethality or severe developmental failure in the injected fish (Fig. A2). Currently, such “monster phenotypes”, are usually interpreted as off-target effects of MOs, and the use of MO in research has been recently criticized (Kok et al., 2015). To minimize the risk of MO off-target effects, we decided to study the effects of MO concentrations that generally did not induce dramatic phenotypes, but with *lin28b* mRNA expression still reduced to approximately less than 10% of the WT expression (1.5 ng, Fig. 2J&K). Most of these *lin28b*-knockout embryos remained WT-like, although some still showed distinct phenotypes that could not be verified to result specifically from *lin28b* knockdown (Fig. A2). Lowering the MO concentration further to 0.75 ng induced no visible effects in the majority of the embryos, despite a few fish that still showed a reduction in growth (Figs. 2K and 3).

Considering the *lin28b* expression pattern in the larval brain, and previous studies indicating *lin28b* may affect neuron differentiation

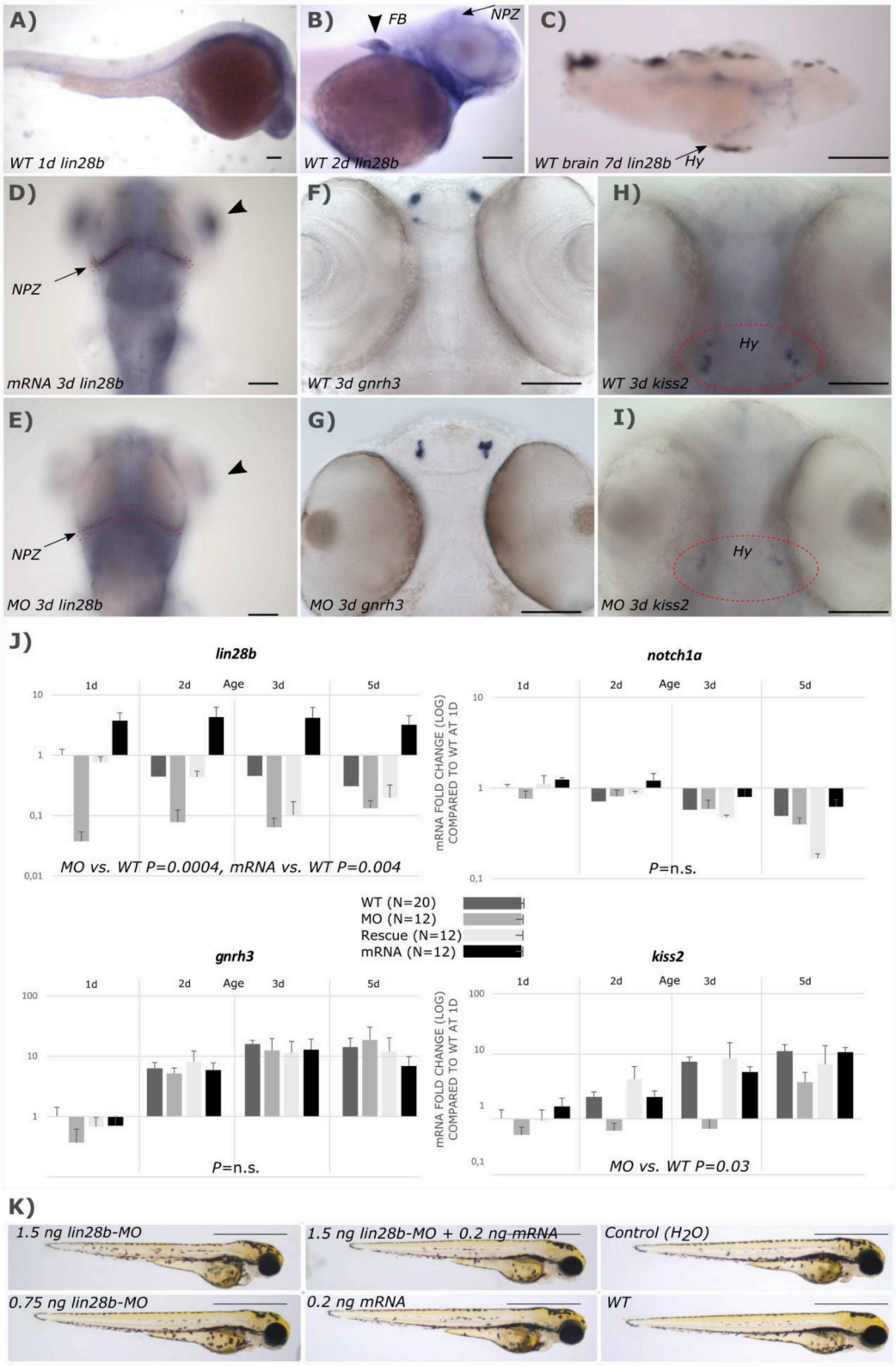

Fig. 2. RNA expression profiling in zebrafish embryos and larvae.

(Balzer et al., 2010), we used qPCR and *in situ* hybridization to assess whether changes in *lin28b* expression might affect neural development related to puberty. To evaluate potential consequences of *lin28b* knockdown on the development of the hypothalamus, we focused on studying *gnrh3* and *kiss2,* homologues of the two human genes *GnRH* and *KISS1* associated with monogenic forms of constitutional delay in growth and puberty (Gajdos et al., 2010; Herbison, 2007). Whereas the *gnrh3*-neurons emerge and migrate from the olfactory placode to the hypothalamus during embryogenesis (Abraham et al., 2008), the *kiss2*-producing neurons are located at the hypothalamus already at 3dpf (Fig. 2H). In contrast to our primary hypothesis that *lin28b* might affect the emergence and development of *gnrh3*-neurons, knocking out *lin28b*

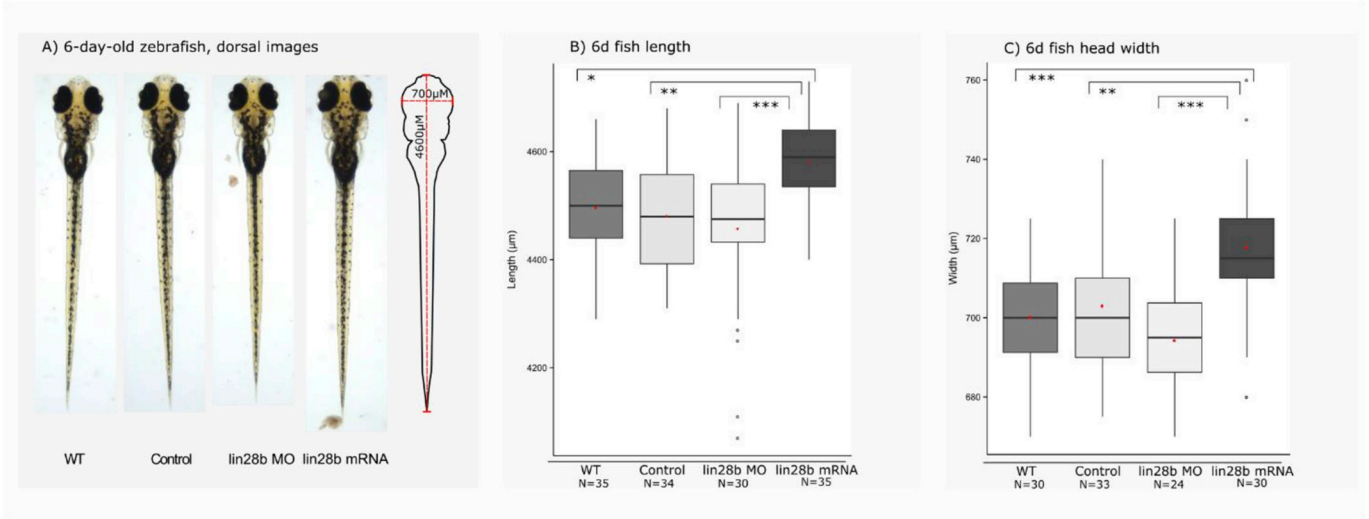

Fig. 3. Comparison of 6d fish size between WT and injected groups. A) Images of 6d fish with scale bars. B) The group injected with *lin28b*-mRNA show significantly longer head-to-tail length, (mRNA vs. WT $P = 0.0069$, mRNA vs. Control $P = 0.0010$ and mRNA vs. MO (0.75 ng) $P = 0.000053$, as well as head width (C) compared to the other groups (mRNA vs. WT $P = 0.000055$, mRNA vs. Control $P = 0.00069$ and mRNA vs MO $P = 6e\text{-}7$, One Way ANOVA with post-hoc Tukey HSD). N = 24–35 per group. Control group was injected with H_20. Although the group injected with 0.75 ng of *lin28b* blocking MO was the only group that contained some visibly smaller fish than others (outlier dots in Fig. 3B), on average the length of these fish was not statistically significant when compared to controls. * = $P < 0.01$, ** = $P < 0.001$, *** = $P < 0.0001$.

did not show any effect on the expression of *gnrh3* mRNA (Fig. 2F&G). Nevertheless, *kiss2* expression, located in the hypothalamus, was significantly affected in the qPCR analysis ($P = 0.033$) and *in situ* hybridization of anti-sense *kiss2* mRNA in 3d embryos (Fig. 2H&I). The RNA expression profiles of embryos injected with both MO and mRNA did not significantly differ from the WT controls (Fig. 2J) which suggests that the effect likely relates directly to *lin28b* knockdown and was not an off-target effect. Similarly, the expression of *notch1a*, a member of a signaling pathway affecting neural progenitor cell maintenance and self-renewal (Louvi and Artavanis-Tsakonas, 2006), remained equal between the MO group and controls, indicating that the knockdown did not result in generally altered brain development that might result from MO off-target effects.

3.4. The effects of lin28b upregulation on embryonic and larval development

Similarly to the knockdown experiments, the injection of high doses of synthetic *lin28b*-mRNA induced visible malformations in most of the injected embryos (Fig. A2). These defects were partly contrary to the ones observed in the knockdown experiments, with some embryos showing relatively dorsalized phenotypes including large heads compared to the rest of the body. However, a more modest dose, still resulting in an average ~4-fold increase in *lin28b* expression, caused no prominent phenotypes during the first days of embryogenesis (Fig. 2K and Fig.A2). After detecting no visible effects from *lin28b* dysregulation during the first days of development, we focused on longer-term effects that might be induced. Intriguingly, already at 6d post fertilization, the fish injected with synthetic mRNA showed significantly increased head-to-tail length ($P = 0.0011$) and head width ($P = 0.00069$) compared to controls (Fig. 3).

3.5. The effect of transient lin28b upregulation on fish growth

Encouraged by the results of 6d larvae injected with *lin28b* mRNA showing generally larger size than their siblings, we decided to continue tracking the growth of the fish throughout juvenile development and into adulthood. Zebrafish are sexually mature after approximately 90–120 days of development, although this is largely dependent on the size of the fish (Chen and Ge, 2013). In an analysis combining data from all time points and both sexes, suggesting that embryonal *lin2*8b expression is crucial for later growth, we observed that the fish whose *lin28b* expression was transiently increased as embryos remained on average longer than their siblings throughout juvenile development ($P = 0.012$, Fig. 4). The result remained significant after adjusting for sex using a logistic regression model ($P = 0.018$). The mRNA injected fish also showed a non-significant trend of increased weight ($P = 0.35$), although the regression analyses suggested this effect was mostly driven by the *lin28b* injected group containing more females than the WT group ($P = 0.042$ for female sex increasing weight, female ratio 50% in mRNA group vs 38% in WT group, Fig. 4).

Having performed the analysis combining all fish at all time points, we proceeded by assessing the effect on growth in more detail. We began by analyzing whether the effect on growth was stronger at specific time points (60d, 90d and 120d). When assessing these time points separately, the fish in the mRNA group appeared significantly longer at 60d and 90d (Welch Two Sample *t*-test $P = 0.019$ and 0.018, respectively), but not at 120d ($P = 0.23$). At 90d, the mRNA group also weighted significantly more than controls ($P = 0.019$, Fig. 4).

We further continued by scrutinizing the sex specificity of the growth effect. Although both sexes generally showed a similar trend of mRNA injection increasing fish size, the effect appeared stronger in females ($P = 0.0002$ for increased female length over the juvenile growth period and $P = 0.038$ and 0.010 at 60 and 120d, respectively, Fig.A3A). In contrast, for males the effect did not reach significance in the respective analyses (Fig.A3B). Interestingly, the effect on growth seemed especially attenuated in males at 120d, around the timing of puberty for most fish in our experiments.

A crucial feature making female zebrafish on average heavier than males is egg production, a definitive marker of sexual maturation in females. Interestingly, we observed significantly more egg carrying fish in the *lin28b* group than in the WT controls (18 vs. 6, $P = 0.036$, Fischer's exact test), thus associating embryonic *lin28b* overexpression with advanced sexual maturation in female zebrafish.

To probe the potential mechanisms leading to the overall greater size of the *lin28b* mRNA injected fish, we assessed RNA expression in the fish brain at 120d. The mRNA-injected fish showed increased expression of growth-hormone-releasing-hormone ($P = 0.041$) compared to untreated siblings. Although *lin28b* expression in adolescent fish was equivalent between the groups, we additionally noted a tendency for

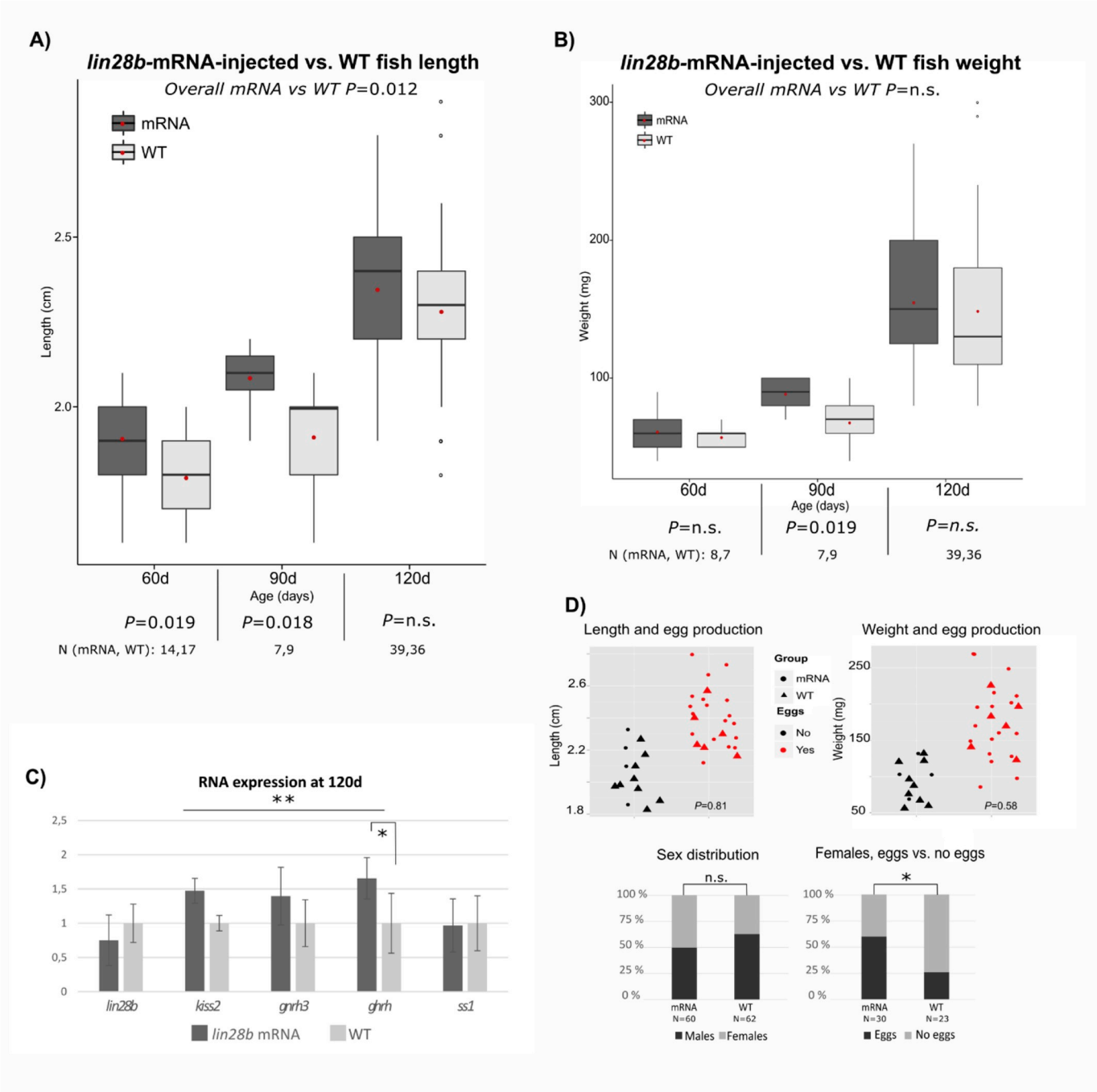

Fig. 4. Comparison of *lin28b* mRNA-injected and wildtype fish size during juvenile growth period (60–120d) A) The *lin2*8b mRNA-injected fish showed on average greater body length ($P = 0.012$, logistic regression analysis adjusting for age, N of WT = 64, N of mRNA injected fish = 61). The result remained significant after adjusting for fish sex ($P = 0.018$). When assessing the effect on length at each time point, significance threshold was reached at 60d and 90d ($P = 0.019$ and 0.018). B) The effect on weight shows a similar trend although not reaching statistical significance ($P = 0.35$, N of WT = 52, N of mRNA injected fish = 54), with female sex increasing fish weight ($P = 0.042$). However, when assessing each time point separately, the mRNA injected fish appeared to weigh significantly more at 90d. C) The mRNA injected fish showed higher expression of *ghrh* and overall greater expression of mRNAs expressed from the arcuate nucleus of hypothalamus (*kiss2*, *gnrh3* and *ghrh*) compared to WT (*$P = 0.041$, and **$P = 0.0011$, One-Way-ANOVA with Post-hoc Tukey HSD, Error bars = SEM). D) Overall, the size of *lin28b* mRNA-injected or WT fish at egg production appeared similar ($P = 0.81$ for length and 0.58 for weight). The mRNA injected population contained more females (50%) than the WT group (38%). The mRNA group also contained significantly more egg producing fish than the WT group ($P = 0.037$, Fisher's exact test). mRNA = fish injected with synthetic *lin28b* mRNA as embryos.

generally increased expression of hormones released from the arcuate nucleus of the hypothalamus in the fish injected with *lin28b* mRNA, including *kiss2, ghrh* and *gnrh3* ($P = 0.0011$).

4. Discussion

The results from the current study highlight the significance of the *LIN28B*-genes as crucial regulators of vertebrate growth, emphasizing both their function in the hypothalamic-pituitary axis (HP) and their importance for successful embryonic and juvenile development. More

specifically, based on transcriptomic data from adult humans, the growth- and puberty-associated polymorphisms in the *LIN28B* region primarily tag changes in the gene's expression levels in the hypothalamus and pituitary. Our results from zebrafish models likewise suggest that the gene has the potential to specifically affect the development of the hypothalamus, and indicates that *LIN28B* function during embryogenesis in part defines the limits for later growth.

4.1. Human data and zebrafish models link lin28b with the function and development of the HP-axis

Notably, our analyses of the GTEx expression database show that the pubertal timing associated SNPs correlate with an increased *LIN28B* expression both in the hypothalamus and the pituitary. We did not observe similar correlations in other tissues, including testis and muscle. This might point towards specific functions of *LIN28B* at the hypothalamic and pituitary level, although it remains possible that the relatively high expression levels in these tissues simply better allowed for EQTl detection. Countering the latter interpretation, in the testis, showing by far the highest *LIN28B* expression of all the tested tissues, the growth-associated SNPs did not show up as EQTls.

GTEx data is based on tissues from adult humans, and although adult tissue may serve as a proxy for exploring the genetic underpinnings of growth and puberty, pre- and peripubertal models can offer more direct clues about the biology underlying these phenotypes. For example, the mechanism underlying Kallman syndrome, a form of hypogonadotrophic hypogonadism, has proven to be deficient migration of *GNRH*-neurons to the hypothalamus during embryogenesis (Gajdos et al., 2010; Herbison, 2007). *lin28b* expression is relatively high throughout this period, and with our zebrafish models we originally wanted to address whether *lin28b* might contribute to the development of the structures essential for puberty, such as *gnrh3 or kiss2*-neurons. The migration of the GnRH-neurons from the olfactory placode to the hypothalamus during embryogenesis appears conserved in all vertebrates, although some inbuilt mechanisms seem to exist to compensate for the loss of *gnrh3* and *kiss2*, and ensure pubertal development in zebrafish (Tang et al., 2015; Smith Spicer et al., 2016). Similarly, the function of kisspeptin, promoting hypophysiotrophic GnRH-secretion due to direct stimulation of GnRH-fibers upon puberty, is well established both in humans as well as in several vertebrate models including zebrafish (Song et al., 2015) Intriguingly, whilst the *gnrh3*-neurons, not yet residing in the hypothalamus, remained seemingly unaffected in our experiments, our data showed that *lin2*8b knockdown may prevent normal development of *kiss2*-expressing hypothalamic neurons. Interestingly, the direction of the effect (less *lin28b* and less *kiss2*) contradicts some previous models suggesting that overexpression of *Lin28a* leads to delayed puberty, and hence likely to later emergence of kisspeptin action (Zhu et al., 2010). Emphasizing the complexity of the *LIN28*-mediated impact on pubertal timing, however, knocking out *Lin28b* in male mice resulted in pubertal delay i.e. an effect of similar direction to the one observed here (Corre et al., 2016). Although the change in *kiss2* neuron development achieved by abrupt knockdown of *lin28b* may not have a direct or major impact on the timing of sexual maturation, it implies that *lin28b* has a broad and diverse potential to affect the function of the HP-axis, and may modify the development of the hypothalamus during vertebrate growth. Nonetheless, caution is warranted when interpreting these results due to the well-known technical challenges associated with morpholino-induced knockdown. Whilst we aimed at controlling for any potential off-target effects, for example the number of the control genes was limited (*notch1a*, *gnrh3,* Fig. 2J), and thus further independent studies are needed to verify the findings.

4.2. The biology behind the phenotypes linked to LIN28B – does timing and tissue matter?

The period of peak expression for *lin28b* - embryogenesis - is the foundation for subsequent growth and development for all organisms. Moreover, epidemiological observations suggest that the predisposition to late onset disease and complex traits in part are linked to growth disturbances occurring during fetal life. Our zebrafish models, in which we cause transient changes in *lin28b* expression during the first few days post fertilization, indeed support that fetal expression of the gene has a long-term effect on overall growth potential. More specifically, transient overexpression of *lin28b* during embryogenesis resulted in a sustained increase of juvenile growth and larger fish size, showing that the influence of the gene on overall growth potential can be determined very early in life. Supporting our findings, the opposite growth effect has previously been associated with *Lin28b* knockdown in mice, i.e. knockdown of *Lin28b* during fetal development, but not later, resulted in decreased juvenile growth (Shinoda et al., 2013a). Nonetheless, while *Lin28b* expression was permanently altered in the mouse model both during the fetal period and also throughout development, our experimental design involved brief dysregulation of gene expression, underscoring that the time window defining growth potential in fact may be very short. An interesting observation was that fish injected with *lin28b* mRNA showed increased *ghrh* expression compared to controls at 120d. Since *ghrh* regulates the secretion of fish growth hormone (*gh1),* this might in theory contribute to the observed increase in fish length. Alternatively, the result may simply be a reflection of the bigger size and more advanced stage of sexual maturation observed in these fish, notwithstanding the actual mechanism underlying the advanced growth.

The other growth phenotype linked to *LIN28*s, besides the length/height growth, is timing of puberty. Whereas egg production is a visible marker of sexual maturation in females, unfortunately we could not identify a similar marker for males and hence we lack data on pubertal timing in male fish. However, in the female fish we somewhat unexpectedly observed that *lin28b* overexpression affected sexual maturation in the opposite direction than shown in human studies. Human GWAS-findings indicate that the same variants associate both with increased childhood growth and delayed onset of puberty, but transient upregulation of *lin28b* during zebrafish embryogenesis resulted in increased juvenile growth and earlier sexual maturation of the fish. The discrepant findings suggest that *lin28b* might have the potential to impact on pubertal timing through separate mechanisms with opposite effects, acting at different times during development. The first mechanism might be more general: for zebrafish, sexual maturation is largely dependent on body size, and any growth advantage is likely to result in earlier maturation, like that achieved by the *lin28b* overexpression in our study (Chen and Ge, 2013). Secondly, the mechanism might be related to the gene's actions later during the juvenile development, namely peripubertal levels of *lin28b* in specific tissues. For example in rats pubertal onset is preceded by a significant decline in hypothalamic *Lin28* expression levels (Sangiao-Alvarellos et al., 2013). Correspondingly, our data shows that at least in zebrafish a transient increase in *lin28b* expression is not enough to reproduce the expected delay in sexual maturation. Moreover, the polymorphisms associating with delayed pubertal timing associate with increased *LIN28B* expression in post-mortem human samples (mostly adult) from the GTEx dataset. Thus we can speculate that the gene's actions closer to the start of sexual maturation might have a stronger impact on puberty than the gene expression patterns during early life. Overall, the accumulating data suggest that *lin28b* might have complex potential to affect pubertal timing through separate mechanisms, acting at different times during development.

5. Conclusions

Epidemiological data have long implied that intrauterine insults at defined periods of development may have lasting effects on physiology, metabolism, tissue and body systems (Wadhwa et al., 2009). While epigenetic programming of genes or altered gene expression have been suggested to mediate these effects, the specific molecular mechanisms have remained elusive. Our current data show that even transient dysregulation of *lin28b* during embryonic development may result in sustained alteration of subsequent longitudinal growth. Based on our findings, *LIN28B* related genetic pathways may represent one set of genetic mechanisms, which – if perturbed during embryogenesis due to any reason – have the potential to impact the subsequent developmental framework over an organism's life course.

Author contribution statement

JTL, YCC and TT performed experiments and analyzed data. JTL and YCC generated reagents. EW and PP supervised the study. JTL drafted the manuscript. All authors critically evaluated and revised the manuscript.

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Declaration of interests

None.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.mce.2018.09.001.

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