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Combination of *in silico* and *in situ* hybridisation approaches to identify potential *Dll1* associated miRNAs during mouse embryogenesis

Bastian Hoesel [a,b], Zambarlal Bhujabal [a], Gerhard K.H. Przemeck [a], Annerose Kurz-Drexler [c], Daniela M. Vogt Weisenhorn [c], Martin Hrabé de Angelis [a,d], Johannes Beckers [a,d,*]

[a] Institute of Experimental Genetics, Helmholtz Zentrum München (GmbH), Neuherberg, Germany
[b] Division of Animal Sciences, School of Biosciences, University of Nottingham, Sutton Bonington Campus, Loughborough, United Kingdom
[c] Institute of Developmental Genetics, Helmholtz Zentrum München (GmbH), Neuherberg, Germany
[d] Technische Universität München, Center of Life and Food Sciences Weihenstephan, Chair of Experimental Genetics, Freising-Weihenstephan, Germany

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ABSTRACT

MicroRNAs (miRNAs) have regulatory functions during vertebrate embryogenesis. They are short ~21 bp long endogenously expressed single-stranded RNAs, which preferentially bind to complementary sequences in the 3′ untranslated regions (UTR) of mRNAs and typically down-regulate the respective target mRNAs by translational repression or enhanced mRNA degradation. The Notch ligand *Delta-like 1* (*Dll1*) is expressed in a highly dynamic pattern and has pleiotropic functions during embryogenesis and in adult tissues. Here, we report an interspecies *in silico* analysis to identify 16 miRNAs, which potentially bind to the mouse, human and chicken *Dll1* 3′UTRs. To analyze whether these miRNAs could regulate *Dll1* gene expression during somitogenesis and neurogenesis, we performed a systematic whole mount *in situ* hybridisation screen, followed by radioactive *in situ* hybridisation on sections, using LNA modified DNA probes in mouse embryos. We find that 7 miRNAs (miR-34a, miR-103, miR-107, miR-130a, miR-130b, miR-449a and miR-449c) are expressed in developing somites, limbs, restricted regions of the brain and neural tube between 9.5 dpc and 12.5 dpc. This suggests that these miRNAs could possibly target the *Dll1* 3′UTR in these regions. The other miRNAs are not expressed or below the detection limit and thus are unlikely to regulate *Dll1* at the analyzed embryonic stages.

1. Results and discussion

MicroRNAs (miRNAs) are short ~21 bp long, endogenous, single-stranded RNAs, that base-pair to specific sites in the 3′ untranslated regions (UTRs) of protein coding mRNAs, typically leading to translational repression of their respective targets or enhanced mRNA degradation (Bartel, 2004). miRNAs are transcribed from the genome and processed by the RNAse III enzyme Dicer. Complete inactivation of Dicer in mice leads to early embryonic lethality (Bernstein et al., 2003). Later functions for Dicer during mouse embryogenesis have also been shown (Chen et al., 2004; Harris et al., 2006; Zhao et al., 2007), suggesting that miRNAs have essential functions throughout development.

The *Dll1* gene has pleiotropic functions (Brooker et al., 2006; Hrabé de Angelis et al., 1997; Kiernan et al., 2005; Przemeck et al., 2003) and is expressed in a complex and dynamic pattern during mouse embryogenesis (Beckers et al., 1999; Bettenhausen

* Corresponding author at: Institute of Experimental Genetics, Helmholtz Zentrum München (GmbH), Neuherberg, Germany. Tel.: +49 (0) 89 3187 3513; fax: +49 (0) 89 3187 4084.
E-mail address: beckers@helmholtz-muenchen.de (J. Beckers).

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et al., 1995; Morrison et al., 1999). For example, during somitogenesis, proper *Dll1* function depends on precise compartmentalized expression (Teppner et al., 2007). Experimental data suggest that *Dll1* is also a target for miRNA-mediated regulation. It has been shown in *Drosophila* that dmiR-1 targets the *Dll1* orthologue *Dl* (Kwon et al., 2005) and evidence suggests that mmu-miR-1 might regulate *Dll1* expression during cell lineage decision of pluripotent embryonic stem cells (Ivey et al., 2008). To screen for additional miRNAs that could regulate *Dll1* expression in mice, we analyzed the *Dll1* 3′UTR sequence *in silico* to identify potential seed regions for existing miRNAs using the public MicroCosm Targets database (Version 5.1) (Griffiths-Jones et al., 2008).

Since the presence of a seed region alone is not sufficient to predict a functional role for a miRNA, we first did an interspecies comparison of the human, mouse and chicken *Dll1* 3′UTRs to identify evolutionary conserved binding sites. For the selection of miRNAs for *in situ* hybridisation we took it as a requirement that the corresponding miRNA exists in the respective species. With this strategy we expected a higher chance for choosing relevant miRNAs due to a selective pressure on the respective sites that could be indicative for a potential function. We then performed a systematic whole mount *in situ* hybridisation screen of all candidate miRNAs to identify those, which are spatially and temporally co-expressed with the *Dll1* mRNA in mouse embryos at 9.5 dpc (days post coitum) and 10.5 dpc. Selected positive miRNAs from this study where further analyzed by radioactive *in situ* hybridisation on histological sections of 12.5 dpc embryos.

1.1. In silico prediction and conservation of Dll1 miRNA-binding sites

To identify miRNAs which possibly regulate the *Dll1* gene we performed an *in silico* analysis of the human, mouse and chicken *Dll1* 3′UTRs. The human *DLL1* 3′UTR is 674 bases long and contains 20 predicted sites for 51 distinct and described human miRNAs. The mouse *Dll1* 3′UTR is 678 bases long and contains 18 predicted sites for 24 distinct and described mouse miRNAs, whereas the chicken *Dll1* 3′UTR is 486 base pairs long and contains 16 predicted sites for 13 distinct and described chicken miRNAs. We analyzed the human, mouse and chicken *Dll1* 3′UTRs for miRNA-binding sites, which are conserved among these three species. Using this approach we identified 16 candidate miRNAs (miR-15a, miR-15b, miR-34a, miR-34c, miR-103, miR-107, miR-130a, miR-130b, miR-301a, miR-301b, miR-363, miR-362-5p, miR-369-3p, miR-449a, miR-449c and miR-497), which are either conserved in all three species (Fig. 1A) or between human and mouse only (Fig. 1B). Also, the relative position of miRNA target sites in the *Dll1* 3′UTRs was largely conserved between the three species (Fig. 1A and B).

Sites for most of these miRNAs were not predicted *in silico* for the 3′UTRs of the mouse *Dll3* and *Dll4* genes. One site for miR-449a/b/c was predicted in the 3′UTR of the mouse *Jag2* gene and one site for miR-34c was predicted in the 3′UTR of the *Jag1* gene (not shown). We take these observations as evidence that the combination of miRNAs sites predicted *in silico* for the mouse and human *Dll1* 3′UTRs are not generic Notch ligand signatures, but are rather specific for the *Dll1* gene.

In turn, we analyzed which other genes possess predicted sites for miRNAs that we identified as conserved in the 3′UTRs of human and mouse *Dll1*. We find that several genes involved in Delta/Notch signaling, such as *Wnt3a* (Dunty et al., 2008), *Pax9* (Mansouri et al., 2000), *Neurogenin1* (Ma et al., 1998), *Fgf18* (Hajihosseini and Heath, 2002) and *Jagged2* (Lan et al., 1997), carry predicted miRNA sites that are also present in the *Dll1* 3′UTR (Supplementary Table 1).

1.2. Expression patterns of candidate miRNAs in mouse embryos

To identify miRNAs, which are spatially and temporally co-expressed with the *Dll1* mRNA in the same embryonic tissues at 9.5 dpc and 10.5 dpc (Figs. 2C, 3E, 5C, respectively), we performed a systematic whole mount *in situ* hybridisation screen using single (SL) and/or double (DL) DIG labeled and LNA modified DNA probes for the respective miRNAs.

The published sequence of the miR-1 *in situ* probe (Kloosterman et al., 2006) was used as positive control and shows expression in the heart and myotomes of 9.5 and 10.5 dpc mouse embryos (Figs. 2A and B and 3A and B, respectively). A scrambled probe was used as negative control and showed no expression except a weak staining in the neural tube of 9.5 dpc embryos (Fig. 2D and E, SL and DL probes, dashed arrows, respectively). This weak staining in the neural tube was present to some extent in all 9.5 dpc embryos (but not in older embryos) hybridized with any LNA modified probe (Fig. 2A and B, SL and DL probes, dashed arrows and data not shown) and is thus most likely due to unspecific staining, which is not indicative of genuine miRNA expression. In addition, similar background staining in neural tubes has been reported by others, for example, in chicken embryos also using LNA modified DNA probes (Sweetman et al., 2006).

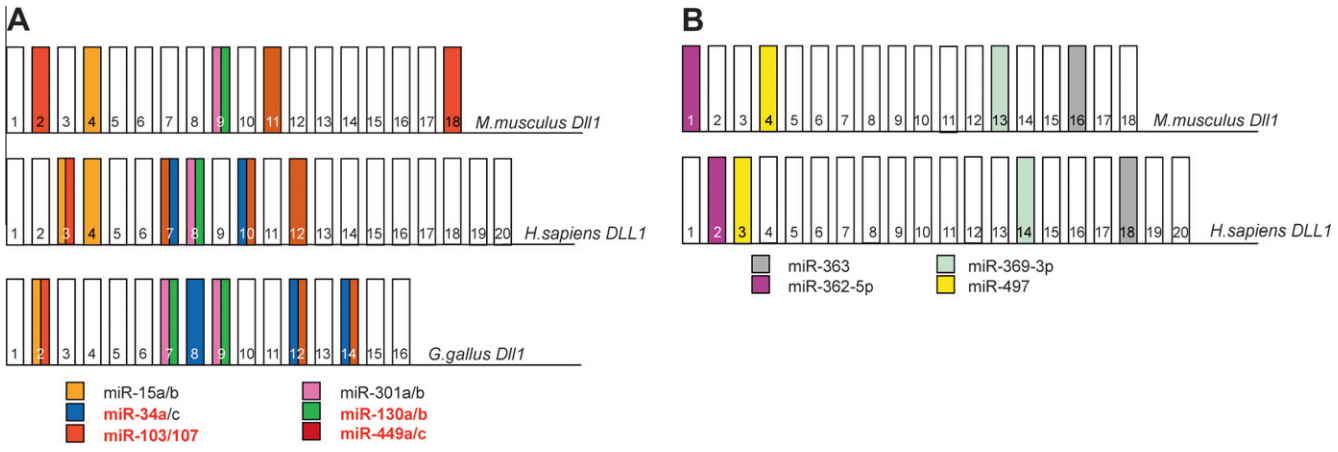

Fig. 1. (A) Alignment of the mouse (top) and human (middle) and chicken (bottom) *Dll1* 3′UTRs, showing conserved miRNA-binding sites indicated by colored boxes. The relative order of the miRNA-binding sites is highly conserved between the three species. (B) Alignment of the mouse (top) and human (bottom) *Dll1* 3′UTRs showing conserved miRNA-binding sites, which are conserved between man and mouse, but not in chicken. miRNAs which are spatially and temporally co-expressed with *Dll1* are marked in red.