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Research paper

Analysis of the *Fam181* gene family during mouse development reveals distinct strain-specific expression patterns, suggesting a role in nervous system development and function

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ABSTRACT

During somitogenesis differential gene expression can be observed for so-called cyclic genes, which display expression changes with a periodicity of 120 min in the mouse. In screens to identify novel cyclic genes in murine embryos, *Fam181b* was predicted to be an oscillating gene in the presomitic mesoderm (psm). This gene, and its closely related paralog *Fam181a*, belong to the thus far uncharacterized *Fam181* gene family.
Here we describe the expression of *Fam181b* and *Fam181a* during murine embryonic development. In addition, we confirm oscillation of *Fam181b* in the psm in-phase with targets of, and regulated by, Notch signaling. *Fam181b* expression in the psm, as well as in the lateral plate mesoderm, was found to be affected by genetic background. We show that *Fam181a* and *b* exhibit partially overlapping mRNA expression patterns, and encode for proteins containing highly-conserved motifs, which predominantly localize to the nucleus. A *Fam181b* loss-of-function model was generated and found to result in no obvious phenotype.

1. Introduction

During development of a complex multicellular organism, the cells which are being constantly generated require temporal and spatial instructions to ensure their correct positioning within the final body structure. Throughout embryonic development, cohorts of cells are instructed to collectively adjust their expression profiles, and thus commit and differentiate into tissues and organs. These changes in expression can occur at regularly spaced intervals — as in vertebrate segmentation. This process, called somitogenesis, leads to the bilateral generation of somites from the anterior end of the presomitic mesoderm (psm). The amount of time required for one somitogenic cycle is species-specific. In zebrafish, a somite pair buds off from the psm every 30 min, in chicken every 90 min, and every 120 min in the mouse. The molecular basis for somitogenesis is provided by morphogen gradients, which confer spatial information to the cells (Aulehla et al., 2003; Dubrulle et al., 2001; Del Corral et al., 2003; Moreno and Kintner, 2004), along with a molecular oscillator termed the segmentation clock. This ensures the correct spatiotemporal formation of somites (Cooke and Zeeman, 1976).

In 1997, Palmeirim and colleagues provided evidence for the existence of the segmentation clock on a molecular level. They showed that changes in the expression of the *c-hairy1* gene in the psm were coordinated with somite formation in the developing chicken at 90 min

Abbreviations: aa, amino acid; BAC, bacterial artificial chromosome; BLAST, basic local alignment search tool; bp, base pairs; cDNA, complementary DNA; cf, compare figure; *c-hairy*, chick *hairy* homolog 1; CMV-Cre, cytomegalovirus promoter driven Cre recombinase; CO_2, carbon dioxide; CpG-methylation, cytosine–guanine dinucleotide-methylation; DAPI, 4′,6-diamidino-2-phenylindole; DIG, digoxigenin; *Dkk1*, dickkopf homolog 1; *Dll1*, delta-like 1; DMEM, Dulbecco's modified eagle medium; DNA, deoxyribonucleic acid; DTT, dithiothreitol; E, embryonic day; EDTA, ethylenediaminetetraacetic acid; EmGFP, emerald GFP; ES cell (ESC), embryonic stem cell; *Fam181a*, family with sequence similarity 181, member A; *Fam181b*, family with sequence similarity 181, member B; FCS, fetal calf serum; FGF, fibroblast growth factor; Fig., figure; FITC, fluorescein isothiocyanate; FlpE, flippase enhanced; GFP, green fluorescent protein; HEK293 cells, human embryonic kidney 293 cells; *Hes1*, *hairy and enhancer of split 1*; ID, identity; IgG, immunoglobulin G; kb, kilo bases; kDa, kilo dalton; *Lfng*, lunatic fringe; loxP, locus of crossover P1; lpm, lateral plate mesoderm; MAMEP, molecular anatomy of the mouse embryo project; MAPK, mitogen activated protein kinase; min, minutes; mM, millimolar; mRNA, messenger RNA; μm, micrometer; NaCl, sodium chloride; NP cells, neural progenitor cells; ORF, open reading frame; ov, otic vesicle; PBS, phosphate buffered saline; PCR, polymerase chain reaction; PFA, paraformaldehyde; *Pmm2*, phosphomannomutase 2; psm, presomitic mesoderm; qPCR, quantitative PCR; RNA, ribonucleic acid; RT-PCR, reverse transcriptase-PCR; S, somite; T7, T7 RNA polymerase; TEAD4, TEA domain family member 4; TS, Theiler stage; U, unit; v/v, volume/volume; WISH, whole-mount *in situ* hybridization; Wnt, wingless-type MMTV integration site family; wt, wild type; YAP1, yes-associated protein 1.

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intervals (Palmeirim et al., 1997). Since then, a number of additional "cycling genes" have been discovered in various species. These have been found to exclusively be targets of either the Notch-Dll (Palmeirim et al., 1997), the canonical Wnt (Aulehla et al., 2003), or the FGF-MAPK signaling pathways (Dequéant et al., 2006).

Recently, Dequéant et al. (2006) used a microarray-based screen of temporally-aligned mouse embryonic psm samples to perform a large-scale search for novel oscillating genes. Both in that study, and in a similar screen performed in our lab (P. Grote, L. Wittler, M. Werber, and B.G. Herrmann, unpublished data), the thus-far uncharacterized gene *Fam181b* (synonym *A830059I20Rik*) was identified as an oscillating transcript with a possible function during segmentation.

The intron-less *Fam181b* gene is located on mouse chromosome 7 and is predicted to encode a protein with a length of 417 aa (~42 kDa). It has one paralog, *Fam181a* (synonym *EG544888*), located on mouse chromosome 12, which encodes a protein of 292 aa (~32 kDa). In this study we analyze the expression patterns of *Fam181a* and *Fam181b* during murine embryonic development and in adult organs, and present initial investigations into the function of the gene family members, thus providing the first comprehensive characterization of the murine *Fam181* gene family.

2. Materials and methods

2.1. Whole-mount in situ hybridization and vibratome sectioning

For whole-mount *in situ* hybridization (WISH), embryos were processed according to the protocol provided by the MAMEP database (http://mamep.molgen.mpg.de). The probe for *Fam181b* corresponds to nucleotides 882–1813 of NM_021427.2, and the probe for *Fam181a* to nucleotides 606–1343 of NM_001195726.1. Probe templates were produced by PCR with a reverse primer containing a T7 site for antisense transcription. DIG-labeled probes were generated by *in vitro* transcription according to standard procedures, and staining was performed using BM Purple (Roche). Following the staining reaction, samples were postfixed in 4% PFA/PBS overnight. Some specimens were used to generate vibratome sections (35 μm thickness) following a sucrose gradient and embedding in a glycerin/albumin matrix.

2.2. In situ hybridization on paraffin sections

Embryos were fixed in 4% PFA/PBS overnight, processed into paraffin wax by standard procedures and sectioned using a microtome (5 μM). *In situ* hybridization was performed on the sections according to the protocol from Chotteau-Lelièvre et al. (2006), with minor modifications. The staining reaction was performed using BM Purple (Roche). For each stage examined at least 3 sections from 2 different embryos were analyzed. There was no observed variation in the staining pattern, and figures show representative staining.

2.3. Tail half cultures

For tail half culture experiments, E9.5 mouse embryos were dissected into ice-cold PBS and their caudal ends bisected along the neural tube using a tungsten needle, leaving several somites anterior to the psm. After incubation of both halves for 30 min in DMEM/F12/10% FCS at 37 °C/7.5% CO_2, one half was fixed in 4% PFA/PBS, while the second half was further incubated for 90 min or 120 min prior to fixation. Corresponding halves were then processed simultaneously for WISH as described above. For comparisons of gene expression at the same timepoint, both halves were immediately fixed after bisection.

2.4. Generation of Fam181b-V5 knock-in and knock-out embryos

To generate a knock-in vector, the genomic region containing the *Fam181b* transcript and a 2.6 kb 3′ homology arm were amplified by PCR from the RP23-168D4 BAC (BACPAC Resources Center, Oakland, CA, USA). The 3′ homology arm contained a repeat of the last 139 bp of the transcript at its 5′ end added by the PCR primer. The V5-tag was inserted at the 3′ end of the *Fam181b* ORF by fusion-PCR. Both modified transcript and homology arm were then inserted into the PL451 vector ((Pgk): Frt-Pgk-em7-Neo-Frt-loxP) (Liu et al., 2003) upstream of the floxed PGK-Neo cassette. For the knock-out vector, a 2.9 kb fragment upstream of the *Fam181b* transcriptional start site was amplified adding a loxP site to the 3′ end. This served as 5′ homology arm and was subcloned, together with the *Fam181b* transcript coding region and the 3′ homology arm, into the PL451 vector upstream of the floxed PGK-Neo cassette. Linearized vector for either the knock-in or knock-out constructs was then used for targeted integration into the *Fam181b* locus of G4 mouse embryonic stem (ES) cells (129S6/SvEv × C57BL/6 N background), and correct integration was verified by Southern blot. For the knock-in, the selection cassette was subsequently removed by transient transfection of positively targeted ESCs with a FlpE-containing expression plasmid. Negative selection and Southern blot verified loss of the cassette. Highly chimeric embryos were generated (70–80% chimerism) by morula aggregation (Eakin and Hadjantonakis, 2006). To generate knock-out animals, chimeric F0 animals were directly crossed to *CMV-Cre* animals (C57BL/6 J background) for deletion of the transcript and the selection cassette. The offspring were then intercrossed, and the resulting embryos/animals used for analysis. All animal procedures were performed in ethical accordance with protocols set out by the Max Planck Institute for Molecular Genetics, with prior approval of the Berlin Animal Welfare Authorities (LAGeSo).

2.5. Differentiation of ESCs along the neural lineage

Murine G4 ESCs were grown under feeder-free conditions and subjected to *in vitro* differentiation into glutamatergic neurons according to the protocol established by Bibel et al. (2007). Samples were taken every 2 days after the formation of cellular aggregates.

2.6. RNA extraction, cDNA synthesis, and quantitative PCR

For RNA extraction, samples were lysed in TRIzol® Reagent (Life Technologies). Total RNA was extracted using the RNeasy Plus Mini Kit (Qiagen) and transcribed into cDNA using the QuantiTect® Reverse Transcription Kit (Qiagen), both according to the manufacturer's protocols. For real-time quantitative PCR, cDNA and appropriate primer pairs were combined with GoTaq® qPCR Master Mix (Promega) and run on the StepOnePlus Real-Time PCR System (Life Technologies). Analysis was performed using either the StepOne software v2.3 (Life Technologies) or the ΔΔct method and q-gene (Muller et al., 2002). P-values were calculated using a one-tailed, paired Student's t-test. For semi-quantitative PCR (RT-PCR), cDNA was used in a standard PCR reaction with GoTaq® Flexi DNA polymerase (Promega). The following mouse-specific primers were used: *Fam181a* fwd: cctatcccgactaagccagc/*Fam181a* rev: gccaaaagagagagggctga; *Fam181b* fwd: cttcccagattgtgcgttgc/*Fam181b* rev: tctccagaggctggggtaaa; *Oct4* fwd: tgttcccgtcactgctctgg/*Oct4* rev: ttgccttggctcacagcatc; *Pax6* fwd: catggcaaacaacctgcctatg/*Pax6* rev: gcacgagtatgaggaggtctgac; *TrkB* fwd: agcagccctggtatcagcta/*TrkB* rev: cttgatgttcttccgggtgt; *Lfng* fwd: ctgca ccattggctacattg/*Lfng* rev: tgctgcaggttctctaggtg; *Pmm2* fwd: agggaaaggcc tcacgttct/*Pmm2* rev: aataccgcttatcccatccttca; *Gapdh* fwd: tcaagaaggtggt gaagcag/*Gapdh* rev: accaccctgttgctgtagcc.

2.7. Transient transfection, immunofluorescence, and immunoblotting

Transient transfection of NIH3T3, HEK293, and C2C12 cells (ATCC Germany) was performed using Lipofectamine™ 2000 reagent (Life Technologies) according to the manufacturer's instructions. Detection of proteins was performed using an α-V5 primary antibody (Life Technologies; R960-25) at a 1:1000 dilution, or an α-GFP antibody (Life