Myocardial m^{6}A regulators in postnatal development: effect of sex

Dmytro Semenovykh[1,2], Daniel Benak[1,2], Kristyna Holzerova[1], Barbora Cerna[2], Petr Telensky[2,3], Tereza Vavrikova[1,4], Frantisek Kolar[1], Jan Neckar[1], Marketa Hlavackova[1,*]

[1]Laboratory of Developmental Cardiology, Institute of Physiology of the Czech Academy of Sciences, Prague, Czech Republic

[2]Department of Physiology, Faculty of Science, Charles University, Prague, Czech Republic

[3]International Clinical Research Center of St. Anne‘s University Hospital Brno, Dementia Research Group, Brno, Czech Republic

[4]Second Faculty of Medicine, Charles University, Prague, Czech Republic

*Corresponding author: Marketa Hlavackova, Laboratory of Developmental Cardiology, Institute of Physiology of the Czech Academy of Sciences, Videnska 1083, 14220 Prague, Czech Republic. E-mail: marketa.hlavackova@fgu.cas.cz

Summary

N^6-methyladenosine (m^{6}A) is an abundant mRNA modification affecting mRNA stability and protein expression. It is a highly dynamic process, and its outcomes during postnatal heart development are poorly understood. Here we studied m^{6}A machinery in the left ventricular (LV) myocardium of Fisher344 male and female rats (postnatal days one to ninety; P1-P90) using Western Blot. A downward pattern of target protein levels (demethylases FTO and ALKBH5, methyltransferase METTL3, reader YTHDF2) was revealed in male and female rat LVs during postnatal development. On P1, the FTO protein level was significantly higher in male LVs compared to females.

Keywords

Epitranscriptomics, N^6-methyladenosine, Postnatal development, Heart

Epigenetic changes have significant importance during both heart development and the manifestation of heart diseases [1]. However, the role of epitranscriptomics, RNA epigenetics, has not yet been sufficiently explored in this area.

N^6-methyladenosine (m^6A) is the most prevalent internal chemical mark in mRNA. It is a dynamic and reversible modification that regulates RNA splicing, export from the nucleus, stability, and degradation [2]. The deposition of m^6A methylation is mediated by proteins called "writers". The most prominent one is methyltransferase-like 3 (METTL3), the catalytic subunit of a multicomponent methyltransferase complex [3]. In contrast, fat mass and obesity-associated protein (FTO) and alkB homolog 5 (ALKBH5) are "erasers" with the principal function of removing the m^6A modification [4,5]. Besides m^6A, FTO also demethylates m^6Am, the main target of FTO in the cytosol, and N^1-methyladenosine (m^1A) in tRNA [6]. The biological functions of m^6A are mediated by "readers" that bind to m^6A-containing RNAs. YTH domain family 1-3 (YTHDF1-3) proteins are eminent m^6A readers that all induce mRNA degradation [7]. The expression patterns of YTHDF paralogs differ across different cell types and tissues. Therefore, the dominant decay-inducing role is usually carried by the most abundant reader in particular cells. Importantly, YTHDF2 is often more highly expressed than YTHDF1 or YTHDF3 [7]. The m^6A modification seems to have significant importance in the developing heart. Disruption in the proper functionality of m^6A machinery proteins can lead to critical alterations in heart structure and function. For example, loss of enzymatic activity of FTO can lead to a ventricular septal defect, atrioventricular defect, and hypertrophic cardiomyopathy in humans [8]. Moreover, according to Su et al. [9], FTO levels drop in elderly murine hearts in response to acute myocardial ischemia/reperfusion injury, while those in young hearts are unaffected. The function of ALKBH5 is linked with an improvement in cardiac function and regeneration after myocardial infarction in juvenile and adult mice [10]. Recent reports also show progressive alterations in m^6A levels during heart development [10-13]. However, there is a lack of data regarding the detailed m^6A machinery protein profiles in heart tissue during postnatal development and potential sex differences.

This pilot study aimed to investigate sex-specific changes in main m^6A regulatory protein levels during postnatal development.

This study was conducted in accordance with the European Guidelines on Laboratory Animal Care. The use of animals was approved and supervised by the Animal Care and Use Committee of the Institute of Physiology of the Czech Academy of Sciences (No. 66/2021).

Animals: Fischer344 rats used for the experiments were bred and kept in the Faculty of Science of Charles University and sacrificed on postnatal days (P) 1, 4, 7, 10, 12, 14, 18, 21, 25, 28, and 90 with n = 4-12 in each group (Table 1). The higher number of individual samples

in the early postnatal period was used because of their limited size. Rats were housed on a 12 h light/dark regime and were given unrestricted access to food and tap water.

Tissue processing: Hearts were dissected into the right ventricle (RV) and LV with septum and frozen in liquid nitrogen. Due to the limited size of the early postnatal LVs, all samples were grouped considering their age and sex and homogenized in eight volumes of ice-cold homogenization buffer (12.5 mM Tris, 2.5 mM EGTA, 250 mM sucrose, 6 mM β-mercaptoethanol, pH 7.4) with the addition of the protease and phosphatase inhibitor cocktail (Roche Diagnostics, Switzerland) as described previously [14]. The protein concentration (Table 1) was measured using the Bradford assay (Bio-Rad, USA). Protein concentration was significantly lower at P1 compared to other days in both sexes. In males, the protein concentration increased gradually from P1 to P7, while in females there was a dramatic change between P1 and P4. The differences in protein concentration indicate significant changes in the ratio of dry mass to water in heart tissue in the early postnatal period. Our observation is in agreement with the already reported rapid postnatal decline in water content in heart tissue [15].

Immunoblotting: Proteins were separated by SDS-PAGE electrophoresis (10% gels) and transferred to polyvinylidene fluoride (PVDF) membranes (BioRad, USA; 1620177). The membranes were blocked using 5% dry low-fat milk in Tris-buffered saline with Tween 20 (TBST) for 1 h at room temperature and incubated overnight at 4 °C with primary antibodies against: FTO [5-2H10] (Abcam, UK; ab92821, 1:1000), ALKBH5 [EPR18958] (Abcam, UK; ab195377, 1:1500), METTL3 [EPR18810] (Abcam, UK; ab195352, 1:1000), YTHDF2 (Invitrogen, USA; PA5-70853, 1:1000). The membranes were subsequently incubated for 1 h at room temperature with secondary anti-rabbit (Bio-Rad, USA; 170-6515, 1:10000) or anti-mouse (Invitrogen, USA; 31432, 1:10000) antibodies. The chemiluminescence was measured by ChemiDoc™ System (Bio-Rad, USA). Ponceau S staining (Sigma-Aldrich, USA; P7170) was used as a loading control. It was shown as an effective way of normalization of samples of different developmental phases [16]. Both male and female protein levels were expressed as fold change over the corresponding P90 male signal (equal to 1). Female protein levels were recalculated to relevant P90 male signals to enable the quantification of sex-dependent differences.

Statistics: All statistical analyses were performed using GraphPad Prism 8 (GraphPad Software, Inc.). One-way ANOVA with Tukey's multiple comparisons test was used for the assessment of the statistical significance within sex. Two-way ANOVA with Tukey's multiple

comparisons test was used for the assessment of the statistical significance of sex differences. The data were obtained from at least three experiments and are displayed as means ± standard deviation (SD). Results were recognized as statistically significant when $P < 0.05$ (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$).

Protein level profiles of m^{6}A machinery during postnatal development in male and female hearts: To investigate protein levels during postnatal development, we performed a western blot of LV tissue lysates collected from rats on postnatal days 1, 4, 7, 10, 12, 14, 18, 21, 25, 28, and 90. We examined the erasers (FTO, ALKBH5), writer (METTL3), and reader (YTHDF2) proteins of m^{6}A modification. YTHDF2 was chosen because of its highest expression among the paralogs in male LV (with the lowest Cq value indicating the highest gene abundance of *Ythdf2* (25.20 ± 0.47) compared to *Ythdf1* (25.91 ± 0.25) and *Ythdf3* (25.59 ± 0.64)). Firstly, we revealed that the abundance profile of all target proteins had a decreasing pattern during postnatal development (P1-P90) (Fig. 1). ALKBH5 and YTHDF2 declined dramatically between P1-P4 with further indistinct changes in protein levels. FTO and METTL3 protein expression dropped gradually throughout the investigated period. Concerning sex-related differences, it was found that the FTO level is significantly higher (by 40.6 ± 21.4%) at P1 in males compared to females.

Our present study provides insights into the dynamics of m^{6}A eraser, writer, and reader protein levels through postnatal development from P1 to P90 in rat left ventricles of both sexes. We showed that all proteins revealed a downward expression pattern with either a dramatic drop during the first critical period from P1 to P4 (ALKBH5 and YTHDF2) or a gradual decline till adulthood (FTO, METTL3). The decreasing patterns of METTL3 and ALKBH5 levels correspond to previously published reports [10,12]. Han et al. [10] utilized a mouse model and analyzed hearts at P1, P7, and P10. They showed the downregulation of protein and gene levels of ALKBH5 throughout this early developmental period. Also, Yang et al. [12] found a higher protein expression of ALKBH5 and METTL3 at P0 than at P7 in the rat heart, while the FTO level remained unchanged. In contrast, Yang et al. [13] found that the METTL3 level in mouse hearts is higher at P7 and P28 compared to P1. FTO protein level revealed a similar decreasing pattern as was observed in our study, its level at P1 was higher than at P7 and P28 [13]. Utilizing the porcine model, Ferenc et al. [17] showed differences in FTO expression between neonatal samples and adult ones in other tissues: skeletal muscle along with the thyroid gland and adipose tissue displayed the higher FTO signal in the neonatal period.

Interestingly, we observed that the FTO protein level in males is higher than in females at P1. The sex-dependent differences provoked by *Fto* level disruption were found in several reports. For example, sex-specific changes in body weight were observed upon overexpression of *Fto* in mice, with females showing a slightly higher weight gain than males [18]. At the time of weaning, both male and female *Fto* knockout mice were about 65% the weight of wild-type and heterozygous littermates. Nevertheless, *Fto* knockout male mice displayed persistent weight loss throughout their life, while female *Fto* knockout tended to make up the weight deficit by adulthood [19]. It may suggest a more significant role of FTO during the embryonic and early neonatal period in males that is in line with our data, where at P1 FTO level was higher in male samples than in female ones.

In conclusion, this study thoroughly assessed the protein levels of m^6A machinery in rat LVs of both sexes during postnatal development. A downward pattern of all target protein levels was revealed in both sexes. Moreover, the FTO protein level was significantly higher in males compared to females on P1.

Conflict of interest

There is no conflict of interest.

Funding

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Table 1. The number of animals in pooled samples and concentration of total protein in samples of male and female rat hearts.

Samples	Number of animals	Concentration (μg/μl)	SD
Males			
P1	11	5.39	0.96
P4	10	8.35	1.46
P7	4	10.81	0.06
P10	4	10.09	0.40
P12	5	10.62	1.25
P14	4	10.58	1.76
P18	5	12.07	0.13
P21	5	11.27	0.30
P25	5	11.92	0.40
P28	5	11.78	0.86
P90	4	13.23	1.85
Females			
P1	9	6.43	0.87
P4	12	11.82	1.92
P7	6	11.54	2.52
P10	6	10.18	1.58
P12	4	10.75	0.60
P14	5	10.47	1.76
P18	5	10.82	1.87
P21	5	10.32	1.44
P25	5	11.73	2.15
P28	5	12.37	0.96
P90	5	11.86	1.44

P – postnatal day; SD – standard deviation

Figure 1. The protein levels of the m^{6}A regulators in male and female rat hearts. A) Immunoblot analysis and multiple comparisons of the immunoblotting data of fat mass and obesity-associated protein (FTO), alkB homolog 5 (ALKBH5), methyltransferase-like 3 (METTL3), and YTHDF2 (YTH domain family 2) in LV tissue homogenates from P1-P90 male rats. B) Representative western blot membranes displaying FTO, ALKBH5, METTL3, and YTHDF2 protein levels in LV tissue homogenates from P1-P90 male rats and the representative total protein Ponceau S staining. C) Immunoblot analysis and multiple comparisons of the immunoblotting data of FTO, ALKBH5, METTL3 and YTHDF2 in LV tissue homogenates from P1-P90 female rats. D) Representative western blot membranes displaying FTO, ALKBH5, METTL3, and YTHDF2 protein levels in LV tissue homogenates from P1-P90 female rats and the representative total protein Ponceau S staining. Homogenates were pooled with n = 4-12 in each group (details in Table 1). All the protein expression levels were normalized to Ponceau S staining. Both male and female protein levels were expressed as fold change over the corresponding P90 male signal (equal to 1). Experiments were performed independently three times. Protein loading was 15 µg. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ (One-way ANOVA; Tukey's multiple comparisons test). $^{\#}P < 0.01$ compared to corresponding P1 males (Two-way ANOVA with Tukey's multiple comparisons test). MWM – molecular weight marker, P – postnatal day, PC – positive control (rat brain).

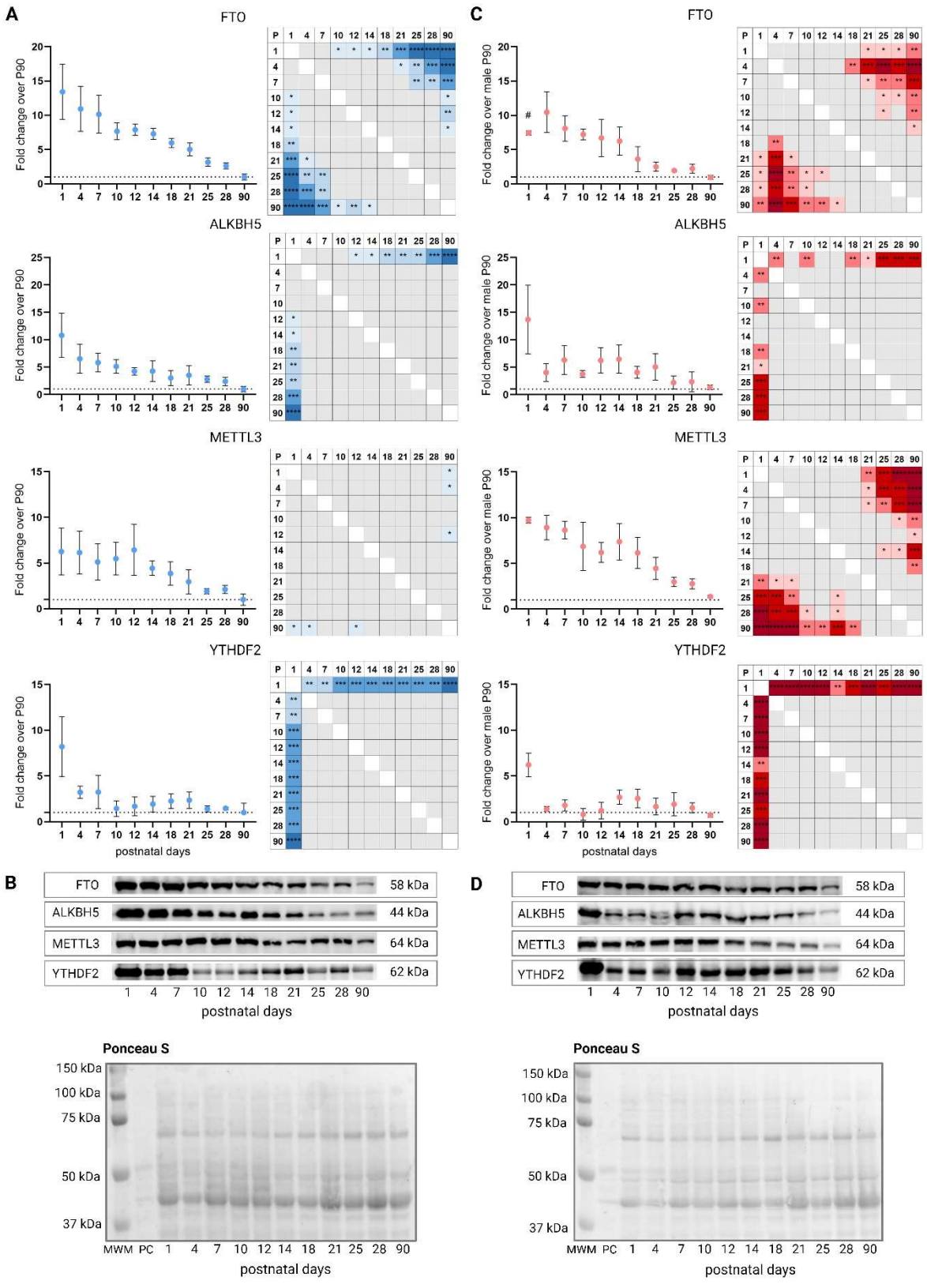
A
FTO
ALKBH5
METTL3
YTHDF2
Fold change over P90
postnatal days
C
Fold change over male P90
B
D
FTO 58 kDa
ALKBH5 44 kDa
METTL3 64 kDa
YTHDF2 62 kDa
postnatal days
Ponceau S
150 kDa
100 kDa
75 kDa
50 kDa
37 kDa
MWM PC 1 4 7 10 12 14 18 21 25 28 90

Fig. 1