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Short communication

Role of let-7b/Fzd4 axis in mitochondrial biogenesis through wnt signaling: In neonatal and adult megakaryocytes

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ABSTRACT

Background: Megakaryocytes (MKs), a rare population of bone marrow cells, are responsible for the production of platelets. Sick neonates are predisposed to developing thrombocytopenia (platelet count <150 × 109/L) and neonates are affected by several megakaryocyte disorders as compared to adults.
Hypothesis: MicroRNAs (miRNAs) have been shown to crucially involve in the regulation of stem-cell differentiation in normal as well as malignant hematopoiesis, but their role in regulation of biological differences between adult and neonatal megakaryopoiesis is unknown.
Methods: To study this, we cultured human cord blood (CB) and peripheral blood (PB) derived $CD34^+$ cells in the presence of thrombopoietin for 14 days and collected cultures expressing >90% $CD41^+$ by flow cytometry and studied 88 miRNAs involved in stem cell development and differentiation. miRNA validation studies were performed in Dami cell line.
Results: Out of 88 miRNAs involved in stem cell development, let-7b was the only miRNA down regulated (~10-fold) in neonates compared to adult-MKs. Let-7b has not been previously described in MKs, however reduced expression of let-7b was found in several human cancers, suggesting that it functions as a tumor suppressor. Our results showed the inhibitory effect of let-7b on wnt signaling pathway by regulating Fzd4 (frizzled family receptor 4) and thereby regulating proliferation as well as differentiation. Let-7b down regulation induced mitochondrial biogenesis and its markers PGC-1α and NRF1 during megakaryocyte development.
Conclusions: Our findings for the first time unveil the novel role of let-7b/Fzd4 axis through wnt signaling by regulating mitochondrial biogenesis during megakaryocyte development.
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1. Introduction

Thrombocytopenia, defined as a platelet count of <150 × 10^9/L, is common among sick infants (Sallmon et al., 2010). Developmental differences between neonates and adult megakaryocytes (MKs) contribute to the vulnerability of neonates to develop severe thrombocytopenia. Although MK development and differentiation follows the same steps in adults and neonates, there are significant differences in ploidy, proliferation and capacity to produce platelets between neonates and adults (Ma et al., 1996; Mattia et al., 2002; Pastos et al., 2006; Sola et al., 2000).

Previous reports suggest the involvement of microenvironment as well as cell-intrinsic factors to these developmental differences (Slayton et al., 2005). Also, the small non-coding microRNAs (miRNAs) are known to play a role with distinct expression pattern during MK differentiation (Ferrer-Marin et al., 2014; Kandi et al., 2014; Raghuwanshi et al., 2015; Undi et al., 2013) (Garzon et al., 2006; Gatsiou et al., 2012). However, the molecular mechanisms governing these differences and the functional role of miRNAs during MK development are not fully understood. The study by Barrey et al. (2011) has shown the existence of miRNA, let-7 in cell organelles, however the role was not fully elucidated (Barrey et al., 2011).

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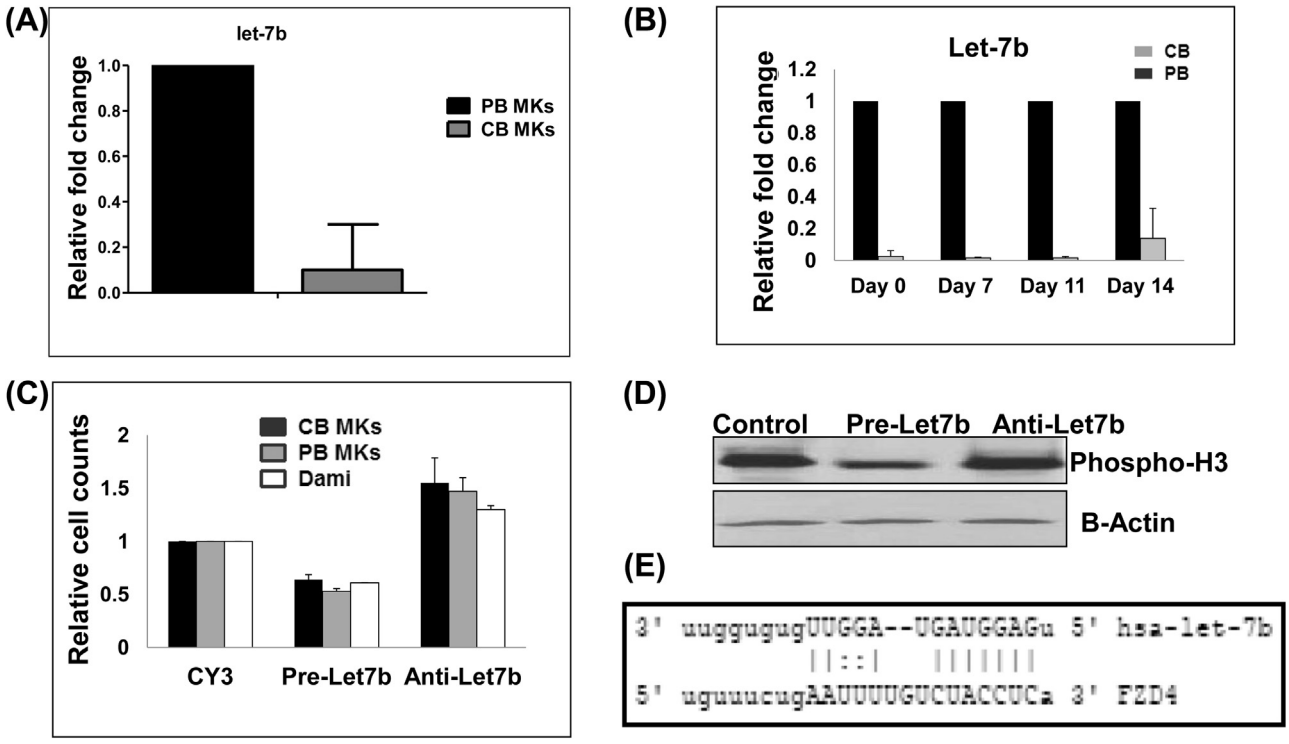

Fig. 1. Expression of Let-7b in CB- vs PB-MKs during MK differentiation. (A) Let-7b levels in CB (grey) and PB-MKs (black) (n = 3, P < 0.05). (B) Let-7b levels in CB cells (grey) at different stages (day 0, 7, 11, 14) of MK differentiation in culture, expressed as relative to miR-99a levels in PB cells (black) at the same stage of differentiation. Bars represent means ± SD of three independent experiments (*p < 0.05). (C) Knockdown and overexpression of Let-7b in primary CB-MKs, PB-MKs and Dami cells results in changing proliferation. Cell counts 48h after nucleofection with pre-let-7b or anti-let-7b, expressed as relative to Cy3 (control). MKs derived from CB (black bars), adult PB (grey bars) and Dami (white bars) were nucleofected, with similar results. The bars represent the mean ± SD of three independent experiments. (D) Immunoblot after 48h of nucleofection with pre-let-7b or anti-let-7b to check the cell proliferation (Higher P-H3 levels were consistent with higher proliferative rate of these cells. Actin was used as a loading control). (E) Prediction of FZD4 as let-7b target using miRBase.

This study was undertaken to understand the role of miRNA in adult and neonatal MK development. Our study provided the role of let-7b in human megakaryocytopoiesis, and for the first time identified let-7b as a molecular regulator of organelle biogenesis through wnt signaling in megakaryocyte development.

2. Materials and methods

2.1. Cell cultures

Human cord blood (CB, Cat #2C-101) and mobilized peripheral blood (PB) $CD34^+$ cells were purchased from Lonza (n = 3 for each group) and cultured in serum free IMDM media with thrombopoietin 50 ng/mL (Pastos et al., 2006). After 14 days of culture, >90% of the cells were MKs ($CD41^+$) by flow cytometric analysis. All experiments in this study were approved by the local IEC.

Dami cells provide perfect model to study the process of megakaryopoiesis. These cells were grown in RPMI-1640 medium supplemented with 10% FBS and antibiotic-anti mycotic (Invitrogen). For induction of Wnt signaling, Wnt3a 50 ng (Bionova) ligand was used.

We also evaluated two megakaryocytic cell lines of different developmental origins. Specifically, we compared CMK cells (a human megakaryoblastic cell line derived from a child with DS-AMKL, which contains a GATA-1s mutation) with those in MEG-01 cells (a human megakaryoblastic cell line derived from the bone marrow of an adult patient with chronic myelogenous leukemia, Ph+ chromosome, in blast crisis). The cell cultures in this study were maintained in contamination free environment.

2.2. Nucleofection

Nucleofection of human CB- and PB- MKs was performed using Pre-let7, Anti-let7b and Cy3 control from Qiagen at day 11 of culture using the Amaxa Nucleofector kit and protocol (Cat. No. VPA-1003, Amaxa Inc.) for primary hematopoietic cells. Similarly, Dami cells were nucleofected and cell numbers were recorded. Cy3-labeled Anti-miR Control is a non-targeting negative control for monitoring transfection efficiency during transfection experiments (Approx. 70%). Live cell number was counted using 0.4% Trypan Blue (Sigma) according to the manufacturer's instructions. After 48 h of nucleofection cells were counted for proliferation and prepared the whole cell lysates for protein expression studies with immunoblot.

2.3. Quantitative RT-PCR

Total RNA (including miRNA) was isolated from CB, PB and Dami cells using the miRNeasy mini kit (Qiagen) and the cDNA (Takara Bio Inc.) was synthesized according to the manufacturer's instructions. The real time PCR was carried out using SYBR Green FAST qPCR Master Mix (Kappa Biosystems) using manufacturer's protocol in an ABI step one plus detection system (Applied Biosystems). Specific primers used for quantification are listed and GAPDH was used as an internal control.

miRNA was reverse transcribed by miScript II RT Kit (Qiagen) and the cDNA was quantified by SYBR Green PCR Kit and Let-7b miScript Primer (Qiagen) using ABI Step One plus detection system. Values were normalized against U6, and CB miRNA values were expressed as relative expression compared to PB. This cDNA was also used for screening 88 miRNA involved in Human

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