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Does hepcidin inhibit iron uptake by binding to divalent metal transporter (DMT1)?

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Abstract

Iron is an essential nutrient for many critical functions in the human body and the deficiency or overload of iron in the cells contributes to the cause of many disorders. This is why it is carefully regulated in order to maintain iron homeostasis. DMT1 is the major iron importer across the enterocytes, while Ferroportin is the only known iron exporter [1]. DMT1 has 4 major isoforms which differ in where transcription starts and whether it has an Iron Responsive Element [1-3]. Research has shown that Ferroportin degrades thus losing its function when hepcidin is bound to it. Recent reports have shown that hepcidin reduces the activity of DMT1 in enterocytes before Ferroportin degrades [7, 8]. We are interested in finding out if hepcidin binds DMT1 as observed for Ferroportin. Our results have shown that hepcidin does not directly bind to DMT1, which lead us to query how hepcidin signals to DMT1.

Introduction

In humans, iron is essential for many critical functions but its ability to exist in the ferrous and ferric states can also make it toxic. Many common disorders in the world are caused by deficiency or overload of iron in the cells, which is why iron is carefully regulated in the human body in order to maintain iron homeostasis.

DMT1 (Divalent Metal Transporter 1) is the major protein used for transporting dietary iron into mammalian intestinal cells [1]. It has 4 major isoforms (1A/+IRE, 1A/-IRE, 1B/+IRE, 1B/-IRE), which differ in where the transcription starts (exon 1A or 1B) and whether the transcript includes or excludes an IRE (Iron Responsive Element) which is part of a regulation system [1-3]. On the other hand, Fpn (Ferroportin) which is found in the basolateral surface of the enterocytes is the only known cellular iron exporter [1].

In ~~year~~ 2001, a 25 amino acid hepatic peptide hormone with anti-microbial properties known as Hepc (Hepcidin) was discovered in human urine [4]. It is synthesized in the liver during inflammatory states or when cellular iron levels are elevated and is called the “master regulator” in iron homeostasis as it is the only known protein that regulates the export of iron. Hepc does so by binding to Fpn [1], marking Fpn for degradation, thus losing its function in exporting iron [5, 6]. It is well-established that Hepc binds to Fpn but there are also reports that Hepc reduces the activity of DMT1 in enterocytes before beginning to degrade Fpn [7, 8]. This leads us to wonder if Hepc binds DMT1 as observed for Fpn. In the case that Hepc binds to DMT1, we could identify Hepc not only as an iron export inhibitor but also as an iron import inhibitor; or in the case that it does not bind to DMT1, it could lead to further research regarding the effects of Hepc on the signal transduction to DMT1.

Results

Tagged-hepc decreases the expression of DMT in HEK293 1A+IRE

We used a tagged form called RhoG-Hepc in HEK293 1A+IRE cell line to affect DMT1 by varying the incubation time (30min, 1hr, 2hr).

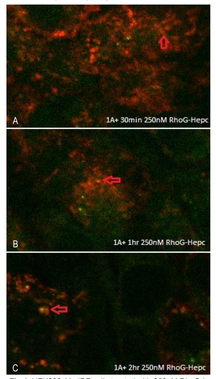
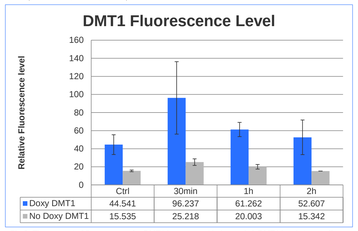


Fig 1. HEK293 1A+IRE cells treated with 250nM RhoG-hepc at 30min, 1hr and 2hr. Red fluorescence corresponds to Exon2 antibodies which binds to DMT1, while green depicts RhoG-hepc. A decrease in DMT1 expression can be seen as the incubation time of RhoG-hepc increases



	Ctrl	30min	1h	2h
Doxy DMT1	44.541	96.237	61.262	52.607
No Doxy DMT1	15.535	25.218	20.003	15.342

Fig 2. The amount of fluorescence for DMT1 is measured in ImageJ. A DMT1 levels taper off as the RhoG-hepc incubation time increases

- There is evidence of co-localization (arrows in Fig 1) even with the weak green signal (RhoG-hepc)
- Level of red signal (DMT1) decreases as the incubation time of RhoG-hepc increases. (Fig 2) (Possibly being ubiquitinated?)

RhoG-hepc might be binding to DMT1 in HEK293 1A+IRE/1B-IRE cells

We wanted to locate the optimal concentration of RhoG-hepc where its level of fluorescence could match Exon2’s better on both HEK293 1A+IRE/1B-IRE cell lines

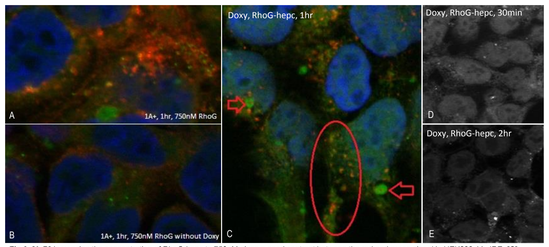


Fig 3. [A-B] Increasing the concentration of RhoG-hepc to 750nM gives a good contrast between the red and green signal in HEK293 1A+IRE. **[C]** Similar results were obtained when the same conditions were applied to HEK293 1B-IRE cells. **[D-E]** In HEK293 1B-IRE cells a decrease in DMT1 expression can also be seen as the incubation time of RhoG-hepc increases

- Increasing RhoG-hepc concentration to 750nM gives a clearer image (Fig 3A), where hints of co-localization are better visualized
- Repeating the same experiment on 1B-IRE cells showed similar results (Fig 3C)
- Comparison between the 30min and 2hr incubation showed a decrease in RhoG-hepc, but with more bright spots (Fig 3D,E)

Pearson’s coefficient	1A+IRE	1B-IRE
Doxy, 500nM RhoG-hepc	0.344	0.336
Exon2 only (positive control)	0.079	0.135

Table 1. Both HEK293 1A+IRE/1B-IRE cells were induced with doxycycline and treated 500nM of RhoG-hepc for 1hr. JACOP (ImageJ plugin) [10] was used to calculate the Pearson’s coefficient between the red (DMT1) and the green signals (Rhog-hepc). Pearson’s coefficient ranges from -1 (negative correlation) to +1 (positive correlation)

- Pearson’s coefficient suggests that there might be a linear relationship between RhoG-hepc in both HEK293 1A+IRE/1B-IRE (Table 1)

There could be a possible hepc binding motif on DMT1

- Identify if there is any possible hepc binding location on DMT1
- Extracted the 19aa (325-342) binding sequence near the human Fpn C326 (Fig 4) [11] and aligned to Rat 1A+IRE
- Identified an partially embedded alignment near tm6 of the Rat 1A+IRE (Fig 5)

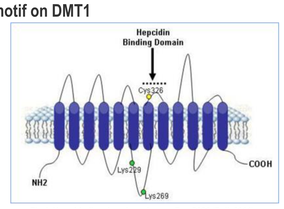


Fig 4. Hepc binding domain on human Fpn C326 (Poli, 2014)

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----------------------------DCITTGYAYTQGLSGSIL-S---- 19
LTFGYEYVTVKPSQSQVLRGMFVPSCSSGGHTFQVEQNGLVGAVIMPHNM 300
                           .* *.    : *: *::: 

-------------------------------------------------- 
YLHSALVKSRQVNRANKQEVKEANKYFFIESCIALFVSFIINVFVVSVFA 350
tm6
```

Fig 5. There is a possible binding motif for hepc near the 6th transmembrane (tm6) domain of DMT1

Results

RhoG-hepc is undetectable in HEK293T-Fpn-GFP cell when its concentration is below 25nM

Find the lowest concentration where RhoG-hepc can still be detected in HEK293T-Fpn-GFP cells which express Fpn inducibly from a ponasterone responsive promoter

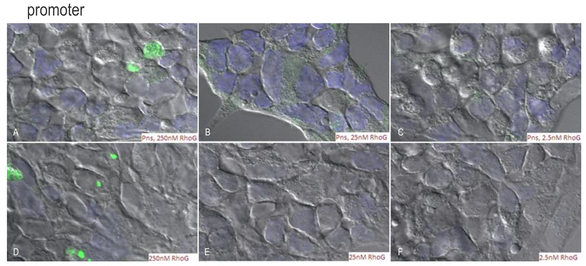

Fig 6. [A-C] HEK293T-Fpn-GFP cells were treated with 20nM of Pns and a 10-fold decrease in RhoG-hepc concentration. **[D-F]** Similar conditions were replicated without the Pns treatment.

- Fluorescence is almost undetectable when it is at 25nM without ponasterone
- GFP signal was detectable but weak, possible conflicts with RhoG-hepc

RhoG-hepc does not have a high affinity to DMT1 in HEK293 1A+IRE/1B-IRE in our western blot

We wanted to see if RhoG-hepc binds to DMT1 on a western blot and since we have more past data for western blots for DMT1. We decided to start the experiment on both 1A+IRE and 1B-IRE cell line.

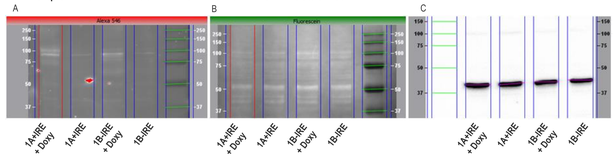

Fig 7. [A] DMT1 is detected with 4EC primary antibody and goat anti-mouse Alexa 568. Bands is detected between 75-100kDa when excited with 546nm light, and there is a distinction for an increased expression of DMT1 when doxycycline is added. **[B]** The blot is stained with RhoG-hepc and scanned with 488nm light. Bands were detected between 37-100kDa with the most intense band residing around ~55kDa. **[C]** Staining with Actin for loading.

- For the cells induced with doxycycline, there is a clearer band when stained with 4EC (anti-DMT1 antibody) (Fig 7A)
- When the blot is stained with RhoG-hepc many bands appeared, with the most intense band residing ~55kDa which is close to the MW of Fpn (Fig 7B)
- There is no distinction of the cells induced with doxycycline for the blot stained with RhoG-hepc, suggesting that RhoG-hepc does not have a high affinity for DMT1 (Fig 7B)

RhoG-hepc may bind to another protein than Fpn in HEK293T-Fpn-GFP

We wanted to see if RhoG-hepc binds to the same location as the Rabbit anti-Fpn antibody on western blot as expected

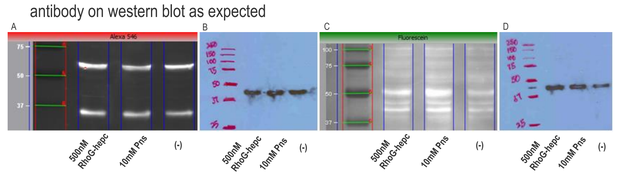

Fig 8. [A] Reagents as indicated were incubated with HEK293T-Fpn-GFP before it is collected. The blot was stained with rabbit anti-Fpn, goat anti-rabbit Alexa 568 and was scanned with a 546nm light. Distinct bands were seen ~35kDa and ~60kDa. [B] Staining with Actin for loading. **[C]** The blot was treated similar to [A] but it was stained with RhoG-hepc instead and scanned with 488nm light. Intense bands were detected ~55kDa and ~40kDa. **[D]** Staining with Actin for loading.

- The rabbit anti-Fpn antibody revealed 2 distinct bands ~35kDa and ~60kDa (Fig 8A), however, the closest band on the blot stained with RhoG-hepc was ~55kDa (Fig 8C)
- The bands on the blot stained with RhoG-hepc did not align well with other bands on the blot stained with rabbit anti-Fpn antibody (Fig 8A, 8C), this leads us to wonder what and why RhoG-hepc binding pattern was out of our expectation

Methods

Cell lines and cell culture: HEK293 1A+IRE/1B-IRE cells contains a TetR:Hyg element originally for β testing [3]. These tetracycline responsive cells were maintained at 37C with 5% CO_2 in DMEM and 10%(v/v) FBS containing 200 µg/ml of hygromycin, 100 µg/ml of streptomycin and 100 units/ml of penicillin. HEK293T-Fpn-GFP cells contains a ecdysone-inducible promoter expressing mouse Fpn with a C-terminal green fluorescent protein (GFP) [9]. These cells were maintained at 37C with 5% CO_2 in DMEM and 10%(v/v) FBS containing 50mg/ml of geneticin, 100mg/ml of Zeocin, 100mg/ml of ciprofloxacin, 100µg/ml of streptomycin and 100units/ml of penicillin. The above cell lines were grown to 70% confluence in ~5-7days and passaged.

Confocal Microscopy: Cells were grown on coverslips and incubated overnight at 37C, 5% CO_2 with doxycycline (HEK293 1A+IRE/1B-IRE) at 10uL/mL or ponasterone (HEK293T-Fpn-GFP) at 10µM or without. RhoG-hepc at varying concentrations were added and incubated at a specified time. They were washed with PBS/1% BSA, fixed with 4% PFA for 10min at room temperature, permeabilized with 0.1% Triton for 5min and blocked with PBS/5% BSA for 20min before incubating overnight at 4C with Exon2 (DMT1 antibody) or Rabbit anti-Fpn antibodies. The cells were stained with Alexa Anti Rabbit, Goat IgG 568, mounted on a glass slide with Prolong Gold with DAPI and imaged with either Zeiss AxioImager Fluorescence Microscope or Zeiss LSM 510 Meta Confocal Microscope.

Western Blot: Proteins were purified from cell extracts by routine methods and then separated by SDS/PAGE and blotted onto nitrocellulose membranes. After blots were blocked in 5% nonfat milk, they were reacted with a 1:1000 dilution of Exon2 or Rabbit anti-Fpn antibody followed by a 1:1500 dilution of Alexa Anti Rabbit, Goat IgG 568 anti-rabbit (secondary) antibody or a 1:2000 dilution of RhoG-hepc. The blots were imaged with BioRad ChemiDoc MP and edited with BioRad Imaging Lab. Blots were subsequently stained with 1:5000 dilution of actin to confirm equal sample loading and efficient transfer.

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