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Iron absorption by Belgrade rat pups during lactation

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Thompson K, Molina RM, Donaghey T, Brain JD, Wessling-Resnick M. Iron absorption by Belgrade rat pups during lactation. *Am J Physiol Gastrointest Liver Physiol* 293: G640–G644, 2007. First published July 19, 2007; doi:10.1152/ajpgi.00153.2007.—Divalent metal transporter-1 (DMT1) mediates dietary nonheme iron absorption. Belgrade (*b*) rats have defective iron metabolism due to a mutation in the DMT1 gene. To examine the role of DMT1 in neonatal iron assimilation, *b/b* and *b/+* pups were cross-fostered to F344 Fischer dams injected with $^{59}FeCl_3$ twice weekly during lactation. Tissue distribution of the radioisotope in the pups was determined at weaning (*day 21*). The *b/b* pups had blood ^{59}Fe levels significantly lower than *b/+* controls but significantly higher ^{59}Fe tissue levels in heart, bone marrow, skeletal muscle, kidney, liver, spleen, stomach, and intestines. To study the pharmacokinetics of nonheme iron absorption at the time of weaning, $^{59}FeCl_3$ was administered to 21-day-old *b/b* and *b/+* rats by intragastric gavage. Blood ^{59}Fe levels measured 5 min to 4 h postgavage were significantly lower in *b/b* rats, consistent with impaired DMT1 function in intestinal iron absorption. Tissue ^{59}Fe levels were also lower in *b/b* rats postgavage. Combined, these data suggest that DMT1 function is not essential for iron assimilation from milk during early development in the rat.

iron transport; lactation; DMT1; Belgrade rat

IRON IS ESSENTIAL FOR THE development and growth of the infant. The neonate receives its iron from milk in the form of nonheme iron (21) that has a high bioavailability (25, 30). The molecular mechanisms responsible for the assimilation of iron from breast milk are not well understood. In the mature intestine, iron uptake across the apical membrane of the intestinal enterocyte is thought to be mediated by divalent metal transporter-1 (DMT1/SLC11A2). Mice with selective inactivation of DMT1 in the intestine (*Slc11a2$^{int/int}$*) have normal hemoglobin and liver nonheme iron levels at birth, but the hematological parameters become reduced by 4 wk of age (15). Iron deficiency becomes even more pronounced after weaning (15). Although the metabolic defects observed in the *Slc11a2$^{int/int}$* mice support a significant role for DMT1 in intestinal iron uptake, whether targeted disruption of the DMT1 gene significantly impairs iron absorption during the early lactational feeding period is unclear. Immunohistochemical analyses have shown that minimal levels of DMT1 protein are present in the mouse intestine at *postnatal days 0* to *5* (24). By *postnatal day 10*, DMT1 becomes localized in the apical membrane of the maturing intestine, but it is predominantly expressed in its deglycosylated/inactive form until *postnatal day 20* (24). In the rat, levels of DMT1 expression continue to increase by *postnatal day 40* (20), but the precise mechanisms responsible for the delivery of milk-bound iron in rats remain poorly understood. For humans, iron absorption from milk is thought to involve lactoferrin (16–18, 21), but whether this milk protein is involved in iron absorption by rat pups is uncertain, particularly since rat enterocytes do not express lactoferrin receptors (17). An alternate pathway for iron uptake mediated by transferrin present in rat milk has been proposed (29). Since DMT1 also plays an established role in transferrin-mediated delivery of iron (11, 13), it would be predicted to function in the latter process.

To better define the function of DMT1 in rat neonatal iron absorption, we have studied the Belgrade (*b*) rat. This animal model of anemia harbors a glycine-to-arginine substitution at codon 185 (G185R) in its DMT1 gene (11). The defective allele encodes a protein with diminished activity in iron uptake assays (1, 7, 8). Belgrade rats have severe defects in iron absorption by several tissues and cell types (8, 10, 13, 19). These animals not only display microcytic anemia but also have defective manganese metabolism due to the apparent role of DMT1 in absorption of both metals (4). We have examined the absorption of iron from breast milk by Belgrade rat pups. ^{59}Fe was administered to lactating foster dams, and the distribution of radioisotope was compared in suckling homozygous and heterozygous Belgrade rat pups at *postnatal day 21*. The results of this investigation indicate that while DMT1 is involved in nonheme iron absorption of the mature intestine of weanling rats, the functional defect of the Belgrade rat's DMT1 gene does not appear to significantly impair ^{59}Fe uptake in the suckling rat pups.

METHODS AND MATERIALS

Animals and diets. Animal protocols were approved by the Harvard Medical Area Animal Care and Use Committee. Belgrade rats were bred from animals obtained from Dr. Michael Garrick (SUNY-Buffalo). Rats were maintained on a 12:12-h light-dark cycle and given food and water ad libitum. Mating pairs of female heterozygotes (*b/+*) and male Belgrade (*b/b*) rats were fed an iron-supplemented diet containing 500 mg Fe/kg (TD02385, Harlan Teklad, Madison, WI; Ref. 33). The breeding and experimental design is diagramed in Fig. 1. Briefly, female *b/+* rats were fed the iron-supplemented diet throughout pregnancy. At *postnatal day 3*, litters of five to seven pups were cross-fostered to F344 Fischer dams (Harlan Sprague Dawley, Indianapolis, IN) fed a standard diet containing 210 mg Fe/kg diet (Purina Laboratories Diet no. 5053, PharmaServ, Framingham, MA). The foster F344 dams were injected twice weekly with $^{59}FeCl_3$, as described below, to examine the transfer of maternal iron during lactation (Fig. 1*A*). For intragastric gavage experiments, a separate litter was cross-fostered but the dams were not injected with isotope

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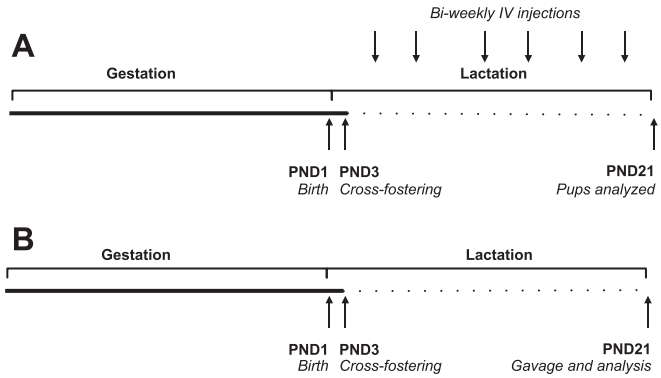

Fig. 1. Breeding and experimental paradigm. Female *b/+* rats and male *b/b* rats were maintained on an iron-supplemented diet containing 500 mg Fe/kg diet during breeding and throughout pregnancy (solid line). At *postnatal day* (PND) *3*, litters were cross-fostered onto F344 Fischer dams fed a standard diet containing 210 mg Fe/kg diet (dashed line). *A*: $^{59}FeCl_3$ was administered twice weekly by intravenous injection during the lactation period (arrows; $n = 2$ litters). *B*: rat pups from a separate litter were administered $^{59}FeCl_3$ on *postnatal day 21* by intragastric gavage (arrow).

(Fig. 1*B*). Homozygous Belgrade (*b/b*) pups are anemic at birth and identified by their pallor; genotype was verified by PCR, as described (11). Serum iron and total iron binding capacity measurements were performed by Kansas State Veterinary Medical Center Comparative Hematology Department (Manhattan, KS).

Pharmacokinetics and tissue uptake of ^{59}Fe. Lactating F344 Fischer dams were anesthetized with vaporized isoflurane prior to intravenous injection of radioisotope into the lateral tail vein. $^{59}FeCl_3$ (Perkin Elmer/NEN, Boston, MA) was diluted in 10 mM ascorbic acid to 75 μCi/ml and injected volumes of 0.5 ml/kg body wt were administered. Pups were humanely killed at *postnatal day 21* by isoflurane overdose and exsanguination, and tissue and blood samples were collected for analyses.

For intragastric gavage experiments, $^{59}FeCl_3$ was diluted in 10 mM ascorbic acid to 30 μCi/ml, and volumes of 1.0 ml/kg body wt were administered to 21-day-old rats through a 19-gauge 4-in. gavage needle. Blood samples were collected from the tail vein at 5, 15, 30 min, and at 1, 2, and 4 h postgavage. Rats were humanely killed 4 h postgavage by isoflurane overdose and exsanguination. Tissue and blood samples were collected and weighed, and radioactivity was measured in a Packard gamma counter (Cobra Quantum, Packard Instrument, IL). Tissue uptake was calculated as total microcuries ^{59}Fe per gram and blood levels were as percent dose. For blood, skeletal muscle, and bone marrow, calculations were based on estimated tissue weight as a fraction of body wt of 7, 45, and 3%, respectively (6). To calculate ^{59}Fe content in blood compartments, the total serum radioactivity (μCi) was determined based on estimated blood weight (6) and red blood cell radioactivity was adjusted for the hematocrit values. Samples are means ± SE [$n = 7$ *b/b* and 5 *b/+* rats; multivariate ANOVA (MANOVA), $^*P < 0.05$].

Immunohistochemistry. Tissue processing and staining for DMT1 were performed according to Canonne-Hergaux et al. (2). Rabbit DMT1 antisera (NH_2 terminal) was a kind gift of Dr. Philippe Gros (McGill University, Montreal, QC, Canada). Following dissection, proximal duodenal sections from normal Sprague-Dawley rats at *postnatal day 1* and *day 21* were rinsed with 2× protease inhibitors (Complete Mini; Roche Diagnostics, Indianapolis, IN) diluted in phosphate-buffered saline (137 mM NaCl, 10 mM phosphate, 2.7 mM KCl, pH 7.4) and then placed in 10% neutral buffered formalin overnight at 4°C, paraffin embedded, and sectioned at 6 μm. Anti-DMT1 (1:250) was visualized by peroxidase-based immunohistochemistry with swine anti-rabbit IgG (1:50) and rabbit peroxidase-anti-peroxidase (1:100) (Dako North America, Carpinteria, CA). Sections were counterstained with nuclear fast red (Sigma, St. Louis, MO). Antibody controls showed no staining, confirming specificity of detection.

Table 1. *Characteristics of b/b and b/+ 21-day-old rat pups*

	b/b	*n*	*b/+*	*n*
Body wt, g	49.636±4.403*	11	83.260±6.464	10
Hematocrit, %	14.642±0.751*	11	37.213±1.271	10
Serum iron, μg/dl	360.570±42.180	7	357.600±31.710	5
Serum TIBC, μg/dl	612.710±14.830*	7	549.400±20.700	5
Serum % Tf sat.	59.750±8.150	7	64.900±4.480	5

Values are means ± SE. TIBC, total iron binding capacity; Tf sat., saturation. $^*P < 0.05$, two-tailed Student's test, assuming unequal variance.

Membrane isolation. Membrane fractions were isolated from the duodena of *postnatal day 1* ($n = 4$) and *postnatal day 21* ($n = 5$) Sprague-Dawley rats (Charles River Laboratories) in the following manner. Duodena were rinsed in protease inhibitors as described above and then homogenized on ice in ice-cold breaking buffer (20 mM HEPES, pH 7.4, 100 mM KCl, 85 mM sucrose, 20 μM EGTA, pH 8.0) containing 2× protease inhibitor (Complete Mini). After centrifugation at 3,500 rpm at 4°C for 10 min, supernatant fractions were centrifuged at 95,000 rpm at 4°C for 10 min. The resulting pellet was washed and resuspended in breaking buffer. Triton X-100 was added to a final concentration of 1%, and samples were gently rotated at 4°C for 1 h to solubilize membrane proteins. After further centrifugation at 95,000 rpm at 4°C for 10 min, the detergent-solubilized protein fraction was collected in the supernatant.

Western blot analysis of duodenal membrane protein. Solubilized duodenal membrane proteins (50 μg) were electrophoresed on a 4–20% gradient SDS-polyacrylamide gel and transferred to Immobilon-Fl polyvinylidene difluoride membrane (Millipore, Billerica, MA) in SDS-free transfer buffer (20 mM Tris, 150 mM glycine, 20% methanol). The membrane was blocked in Tris-buffered saline (TBS; 50 mM Tris, 150 mM NaCl, pH 7.4) containing 5% nonfat dry milk and incubated in rabbit anti-DMT1 antibody diluted 1:1,000 in TBS containing 1% nonfat dry milk. As a control for loading, the immunoblot was also incubated with mouse anti-NaKATPase (DAKO North America) diluted 1:20,000 in the same buffer. Immunoreactive proteins were visualized and quantified using a LI-COR Odyssey Infrared Imaging System with secondary antibodies goat anti-rabbit IR800 and goat anti-mouse IR680 (LI-COR Biosciences, Lincoln, NE).

Statistical analysis. Values reported are means ± SE. Statistical significance was evaluated by MANOVA for all studies by the general

Table 2. *Tissue distribution of ^{59}Fe in b/b and b/+ rat pups after lactation from dams intravenously injected with the radioisotope*

	b/b	*n*	*b/+*	*n*
Blood	429.74±17.60*	7	645.48±29.06	5
Brain	28.98±0.77	7	26.35±0.83	5
Lung + trachea	103.30±6.36	7	91.09±2.79	5
Heart	131.97±6.94*	7	100.72±5.42	5
Bone marrow	71.30±5.40*	7	28.61±1.87	5
Skeletal muscle	21.02±1.78*	7	25.82±1.70	5
Kidney	78.27±5.01*	7	48.02±2.08	5
Liver	108.94±14.92*	7	62.26±2.34	5
Spleen	264.33±20.63*	7	145.13±3.91	5
Stomach	13.72±0.94*	7	9.93±0.83	5
Small intestine	21.08±1.88*	7	10.29±0.65	5
Large intestine	29.92±4.45*	7	9.72±1.42	5

Values are means ± SE expressed as nCi ^{59}Fe/g tissue. $^*P < 0.05$, multivariate ANOVA (MANOVA); PROC MIXED analysis determined no effect of dam.

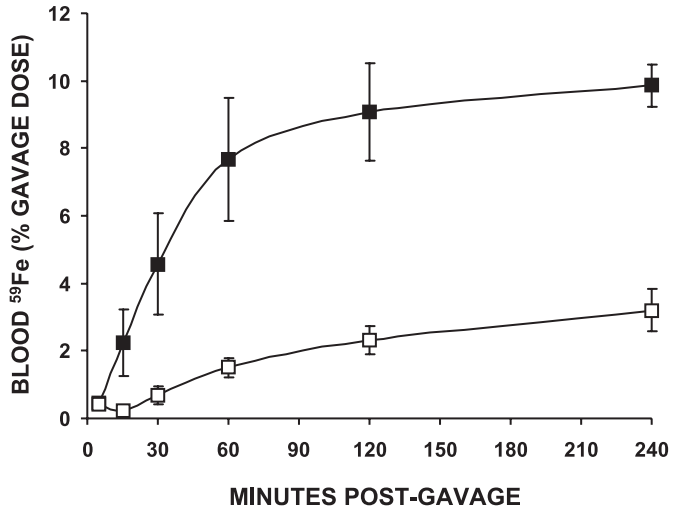

Fig. 2. Pharmacokinetics of intestinal ^{59}Fe absorption in 21-day-old *b/+* and *b/b* rats. Blood samples were collected from the tail vein at 5, 15, 30 min, and at 1, 2 and 4 h after intragastric gavage of *b/+* (■) and *b/b* (□) rats. Samples are mean percentage of gavaged dose ± SE (n = 4 *b/b* and 5 *b/+* rats; multivariate ANOVA, $P < 0.05$).

linear model procedure, and PROC MIXED analysis was performed for the lactation studies on pooled data to test for association of an effect of the dam (SAS statistical analysis software; SAS Institute, Cary, NC). Statistical significance was based on α level of 0.05.

RESULTS

Characteristics of weanling b/b and b/+ rats. The body weight of homozygous Belgrade rats (*b/b*) at the time of weaning (21-day-old) was significantly lower than control heterozygous siblings (*b/+*) (Table 1). The hematocrit values for *b/b* weanlings were extremely low, indicative of the hypochromic microcytic anemia of Belgrade rats (8, 10, 12, 31). Although serum iron levels were not different from *b/+* littermates, total iron binding capacity was significantly increased in *b/b* rats compared with *b/+* rats.

^{59}Fe assimilation by suckling b/b and b/+ rat pups. Lactating foster dams were injected with ^{59}Fe as outlined in Fig. 1*A*, and the distribution of the radioisotope in suckling littermates was compared. The amount of ^{59}Fe in the blood of *b/b* Belgrade rat pups was significantly less than *b/+* siblings (Table 2). Examination of blood compartments from two separate experiments (n = 7 *b/b* and 5 *b/+* rats; $P < 0.05$) showed that ^{59}Fe was elevated in *b/b* serum (54.17 ± 9.20 vs. 27.76 ± 3.27 nCi ^{59}Fe) and reduced in *b/b* red blood cells compared with *b/+* rats (1,145.27 ± 82.79 vs. 2,401.65 ± 38.83 nCi ^{59}Fe). Despite reduced levels of circulating ^{59}Fe, tissue levels of the radioisotope on a per gram basis were elevated in the heart, bone marrow, skeletal muscle, kidney, liver, spleen, stomach, and intestines of *b/b* rat pups (n = 5–7; MANOVA, $P < 0.05$; Table 2). On the basis of body weight, total levels of assimilated ^{59}Fe between *b/b* and *b/+* pups were not different (0.0773 ± 0.0062 vs. 0.0654 ± 0.0026 μCi/g respectively; P = 0.113, two-tailed Student's test, assuming unequal variance).

Table 3. *Tissue distribution of ^{59}Fe in 21-day-old b/b and b/+ rats measured 4 h postgavage*

	b/b	*n*	*b/+*	*n*
Blood	15.98±3.15*	4	49.37±3.10	5
Brain	0.55±0.14*	4	1.50±0.10	5
Lung + trachea	17.12±13.10	4	14.79±1.14	5
Heart	5.09±0.93*	4	12.97±0.92	5
Bone marrow	6.63±1.61*	4	57.76±6.04	5
Skeletal muscle	0.90±0.17*	3	2.13±0.25	5
Kidney	2.13±0.31*	4	9.86±0.88	5
Liver	6.76±1.69*	4	33.52±4.64	5
Spleen	5.09±0.97*	4	166.51±37.47	5
Stomach	649.54±99.46	4	450.34±111.64	5
Small intestine	400.63±62.89*	4	215.54±39.20	5
Large intestine	197.07±45.78	4	223.84±37.98	5

Values are means ± SE expressed as nCi ^{59}Fe/g tissue. *$P < 0.05$, MANOVA.

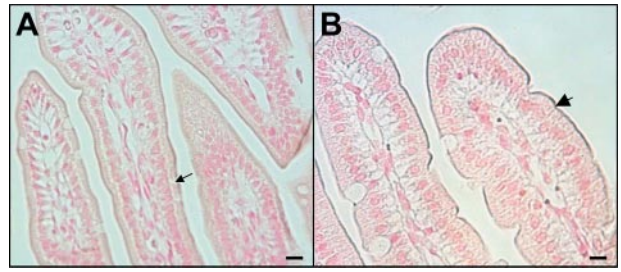

Fig. 3. Divalent metal transporter-1 (DMT1) staining in the developing rat duodenum. DMT1 immunohistochemical staining in the Sprague-Dawley rat duodenum localizes to the cytoplasm (arrow) of the *postnatal day 1* enterocytes (*A*) with slight staining in the brush border, compared with significant brush border staining (arrowhead) in the *postnatal day 21* duodenal enterocytes (*B*). Sections are counterstained with nuclear fast red. Digital images were obtained with a Nikon D100 camera (Nikon, Japan). Images were acquired by iPhoto software [version 5.0.4 (263), Apple software]. Bar = 20 μm.

Pharmacokinetics and tissue distribution of ^{59}Fe after intragastric gavage of 21-day-old weanlings. To examine the role of DMT1 in iron absorption at the time of weaning (21 days), ^{59}Fe was administered by intragastric gavage, and the pharmacokinetics of absorption and tissue distribution of the isotope was determined as outlined in Fig. 1*B*. The absorption of ^{59}Fe from the gut to the blood was significantly impaired in *b/b*

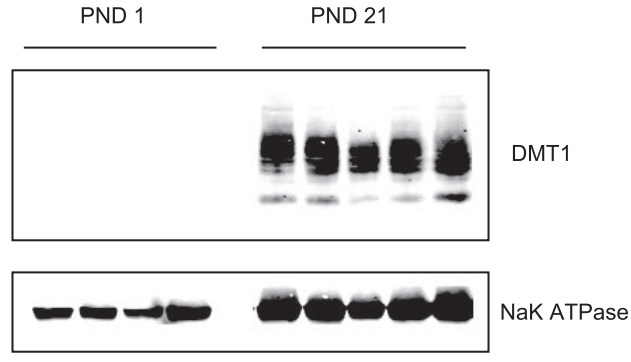

Fig. 4. Western blot analysis of DMT1 in duodenal membrane fractions of 1-day-old and 21-day-old rats. DMT1 was detected in the membranes isolated from *postnatal day 21* (PND 21) rats but not *postnatal day 1* (PND 1) rats. Na-K-ATPase was used as a loading control and was detected in all duodenal samples. Anti-DMT1 antibody was a kind gift of Dr. Philippe Gros (McGill University). Proteins were visualized and quantified by using the LI-COR Odyssey Infrared Imaging System and the LI-COR secondary antibodies goat anti-rabbit IR800 and goat anti-mouse IR680 (LI-COR Biosciences, Lincoln, NE).

weanlings ($n = 4–5$; MANOVA, $P < 0.05$; Fig. 2). Levels of ^{59}Fe circulating in the blood at 4 h were only 32.4% of values measured for *b/+* siblings. ^{59}Fe tissue levels were also decreased in Belgrade *b/b* rats compared with *b/+* rats in the brain, heart, bone marrow, skeletal muscle, kidney, liver, and spleen, but increased in the small intestine ($n = 4–5$; MANOVA, $P < 0.05$; Table 3); these observations are consistent with the significantly reduced intestinal absorption of the *b/b* rats. The reduced uptake of iron administered by intragastric gavage is consistent with previous studies of intestinal iron absorption by Belgrade rats (10, 26–28) and of duodenal extracts from these animals (19).

Intestinal DMT1 expression in the developing rat duodenum. Previous studies have suggested that DMT1 is expressed at low levels immediately after birth but that functional, fully glycosylated, and apically expressed protein is not present until later times of development (20, 24). Immunohistochemical staining for DMT1 was compared in intestinal samples collected from Sprague-Dawley rats on *postnatal days 1* and *21*. As shown in a representative panel in Fig. 3, more intense DMT1 staining was observed at the intestinal brush border at *day 21* compared with *day 1*, and cytoplasmic DMT1 staining was more prominent at *postnatal day 1*. This expression profile for DMT1 in the developing duodenum was further confirmed by Western blot analysis (Fig. 4). DMT1 was not detectable in duodenal membrane preparations from *postnatal day 1* rats but was highly expressed by *postnatal day 21*.

DISCUSSION

DMT1 is known to play a major role in intestinal nonheme iron transport in adult animals (15). However, the mechanisms responsible for the acquisition of iron from milk by the developing rat duodenum, as well as the function of DMT1 in the rat neonatal duodenum, are unclear. Previous reports (20) and observations made in this study (Figs. 3 and 4) show that in the rat intestine DMT1 is present at very low levels at birth but is expressed in its functional form at the brush border at the time of weaning in rats. Our study demonstrates that overall levels of ^{59}Fe absorbed by suckling *b/b* and *b/+* rat pups during the lactation period were remarkably similar, supporting the idea that transport pathways that do not involve DMT1 play a more significant role in iron assimilation from milk. One potentially unexpected result was that significantly greater amounts of the radiotracer ^{59}Fe were found in certain tissues despite defective DMT1 function in the *b/b* rats. Since DMT1 plays a role in iron acquisition from transferrin, these observations suggest that alternate pathways also available to mediate iron uptake by peripheral tissues. This idea has been supported by previous studies showing that liver iron uptake persists in DMT1 knockout mice (15), as well as our own characterization of liver iron loading in the Belgrade rat (33).

In contrast, the pharmacokinetics of ^{59}Fe administered by intragastric gavage show significantly reduced uptake by 21-day-old *b/b* rats compared with *b/+* controls (Fig. 3); thus uptake of nonheme ^{59}Fe is impaired in Belgrade rats at the time of weaning. These combined observations suggest that a developmental transition to DMT1-mediated iron uptake occurs as food intake begins and that other transport mechanisms may be primarily responsible for iron assimilation during early lactational feeding. It is important to keep in mind, however, that there are differences in availability of iron contained in milk vs. dietary iron. Following intake of dietary nonheme iron, it must be reduced from a ferric state to the ferrous state for uptake by DMT1. It is unclear what form of iron is present in milk that affords its high bioavailability. The mechanisms responsible for the delivery of milk-bound iron remain relatively uncharacterized.

Some alternative transport mechanisms for iron uptake by rat enterocytes have been suggested in past studies (29). The rat has high transferrin concentrations in milk (23), and the presence of transferrin receptors has been reported in the rat small intestine (17). Uptake of iron through the transferrin/transferrin receptor complex may occur during early development. However, DMT1 plays an established role in transferrin-mediated delivery of iron, and uptake through this endocytic pathway is impaired in the Belgrade rat owing to the G185R mutation in DMT1 (11). The fact that the overall amounts of iron assimilated by *b/+* and *b/b* suckling rats are remarkably similar argues against a major role for this pathway in early development. Lactoferrin is also thought to contribute to iron acquisition in the neonate (16–18, 21). Humans and monkeys have high levels of lactoferrin, and lactoferrin has been identified in rat mammary gland during the lactation period (36). High levels of lactoferrin receptors have been found in rhesus monkeys (5) and in human fetuses and infants (18). However, it is unclear how bound lactoferrin might be internalized by rat enterocytes (3), which do not appear to express lactoferrin receptors (17). Uptake of infused bovine lactoferrin from the rat intestine to the blood has been shown to occur via the lymphatic pathway (32). Moreover, studies of lactoferrin knockout mice have indicated that neonatal intestinal iron uptake is normal in these animals (35), strongly arguing against a major role for this milk protein in iron assimilation by rodents. Alternative metal transport pathways in the neonate may involve members of the SLC39A zinc transporter family, which have been shown to transport iron and manganese, in addition to zinc (9, 14, 22, 34). Zip14, in particular, is highly expressed in the intestine and has been shown to transport iron (22). Future studies are required to explore these and other alternative pathways for neonatal iron absorption from milk.

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