Milk growth factors and expression of small intestinal growth factor receptors during the perinatal period in mice

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BACKGROUND: Growth factors (GFs) are milk bioactive components contributing to the regulation of neonatal small intestinal maturation, and their receptors on the small intestinal epithelium play essential roles in mediating the functions of GFs. There is limited data correlating milk GFs and their receptors in the neonatal small intestine during the perinatal period.
METHODS: Small intestines of C57BL/6N mouse pups were collected at regular intervals during fetal life and up to postnatal day (PD) 60. Gene expression of GF receptors was determined by real-time qPCR. Milk GF concentrations up to PD21 were analyzed by enzyme-linked immunosorbent assay.
RESULTS: The majority of GF receptors showed significantly greater expression in the fetus than in postnatal life, and a sharp decrease occurred from PD14 extending to PD60; solid food restriction (PD14 and PD18) did not affect this decrease. Concentrations of five detected milk GFs demonstrated that GFs and the corresponding small intestinal receptors exhibited different correlations, with only milk transforming growth factor β1 (TGF-β1) having a significant positive correlation with TGF-β receptor 1 mRNA.
CONCLUSION: Gene expression of small intestinal GF receptors is likely a process of neonatal intestinal maturation that is affected concurrently by milk GFs and additional endogenous factors.

The small intestine is a complex organ that plays a crucial role in infant nutrient absorption. The development of the small intestine is related to many factors, e.g., fluid balance, electrolytes, enzymes, and hormone regulators, and disruption of this balance can lead to neonatal weight loss, growth retardation, and even death (1).

Breast milk contains multiple bioactive components that stimulate growth of the small intestine in infants (2–5). These growth factors are a group of whey proteins, such as epidermal growth factor (EGF), insulin-like growth factor (IGF), hepatocyte growth factor (HGF), and transforming growth factor-β (TGF-β), which regulate intestinal crypt cell proliferation/differentiation. Known functions of growth factors in the small intestine are stimulation of cell growth and regulation of intestinal inflammation. IGF-1 can attenuate apoptosis of intestinal epithelial cells induced by H_2O_2 in rats through the PI3K (phosphoinositide-3-kinase) pathway (6). This anti-apoptosis function of IGF-1 in normal intestinal mucosa is highly dependent on insulin receptor substrate-1 (IRS-1) expression (7). A study in transgenic mice found that IGF-1 stimulated intestinal mucosal growth is partially related to IRS-1 (7). Furthermore, oral administration of TGF-β1 in a rat model with induced necrotizing enterocolitis inhibited NF-κB (nuclear factor-κB) activation by enhancing IκBα expression (8). Gastric cancer cells infected by the pathogen Helicobacter pylori have remarkably decreased TGF-β1 expression (9). At the same time, restored TGF-β1 signaling by Smad7 antisense oligonucleotides in mice with colitis decreased IL-12 (Interleukin-12) and interferon-ϒ levels (10). Both studies demonstrate that restoring the level of TGF-β1 could be a promising way to dampen the inflammatory response of the small intestine.

The effects of growth factors on gastrointestinal development are achieved by binding to their receptors at the cell surface and inducing downstream signaling pathways. That growth factor receptors play important roles in gastrointestinal homeostasis has been well documented. EGF receptor knock-out mice die at birth with aberrant intestinal cell proliferation and differentiation (11). Similarly, cultured fibroblasts from knockout mice with a 50% reduction in IGF-1 receptor grow very slowly (12). Concentrations of growth factors in milk change during lactation, and neonatal gut maturation continues until late weaning (13,14). However, knowledge of the expression patterns of growth factor receptors in the small intestine during the perinatal period is very limited.

We hypothesized that the expression of growth factor receptors in the small intestine changes during the lactation period to allow the infant to adapt to its new environment. This was explored in a mouse model by measuring the levels of EGF receptor (EGFR), fibroblast growth factor receptor (FGFR), hepatocyte growth factor receptor (HGFR), IGF-1 receptor (IGFR), insulin receptor (InsR), nerve growth factor receptor (NGFR), platelet-derived growth factor receptor (PDGFR), TGF-β receptor (TGF-βR) and vascular endothelial growth

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factor receptor (VEGFR) from embryonic day 13 through postnatal day 60.

RESULTS

Growth Factor Receptor Protein Expression in the Small Intestine

Expression of growth factor receptors at the protein level was determined in samples of small intestines collected on PD 0, 3, 5, 7, 10, 14, 18, 21, 30, and 60. EGFR, TGF-βR1 and 2, IGF-1R, and InsR were determined by immunoblotting. EGFR intestinal content increased from birth, reached a maximum at PD10 and started to decrease at PD12. After PD18, it increased again and then stayed at a similar level until PD60. The expression of TGF-β receptors 1 and 2 reached a maximum around PD14 and decreased significantly after PD18. Maximum expression of InsR and IGF-1R occurred at PD10 and PD7, respectively. They all became stably expressed around weaning (**Figure 1**). We noticed that the conventional internal controls for loading such as β-actin, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), β-tubulin did not show similar expression across the lactation period on a per total protein quantity basis, which may be due to the rapid metabolism of neonates.

Growth Factor Receptor Gene Expression in Small Intestine

In our study, GAPDH mRNA was stably expressed in early infancy, and therefore GF receptors were analyzed at the mRNA level using GAPDH mRNA as an internal control. Real time qPCR showed that all receptors assayed including EGFR, FGFRs, HGFR, IGF-1R, InsR, NGFR, PDGFR, TGF-βR1, and VEGFR had maximum expression during gestation at gestational days 13 or 18 except for TGF-βR2, that had maximum expression at PD0 (**Figure 2**). Starting from PD0, expression of all growth factor receptors increased and arrived at a maximum at PD14. A sharp decrease in expression occurred from PD14 to PD18 for all receptors. After weaning at day PD21, expression of the receptors reached a plateau and there were no significant changes between PD21 and PD60.

Solid Food Restriction

To exclude effects from the intake of solid food on gene expression of GF receptors, a food restriction experiment was conducted between PD14 and PD18. RNA of small intestines from pups that had no access to solid food (RI) was collected at PD14, PD16, and PD18. The sample from PD0 was used as a control to make a comparison with results from nonrestricted pups. Although we observed some modest changes in FGFR1, FGFR2, IGF-2R, VEGFR expression at PD14 as well as in IGF-1R and InsR at PD18, solid food restriction did not change the expression pattern for GF receptors during the perinatal period to a significant extent. Similar to the results for samples from normally fed pups (NI), all growth factor receptors had maximum expression at PD14 and decreased significantly at PD18 except for InsR and IGF-1R, which decreased from PD14 to PD18, although the difference was not significant (**Figure 2**).

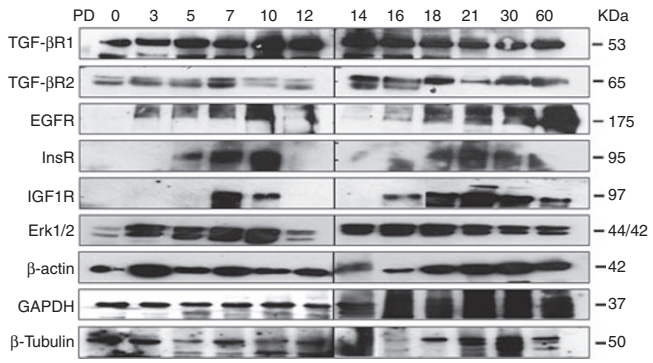

Figure 1. Growth factor receptor protein expression in mouse small intestine. Membrane proteins (50 μg) were extracted from small intestines collected at regular intervals and analyzed by immunoblotting ($n = 1 \times$ (5–8 pups)).

Milk Growth Factor Concentrations and Correlation With Gene Expression of Small Intestinal Receptors

EGF, TGF-β1 and 2, IGF-1, VEGF, and PDGF were analyzed in mouse milk collected at PD 0, 5, 10, 14, 16, 18, and 21. The concentration of EGF was as low as 28 ± 7 ng/ml at PD0, sharply increased during the lactation period, reaching 250 ± 28 ng/ml at PD16. At weaning, it slightly decreased to 153 ± 30 ng/ml. The concentration of TGF-β1 was 907 ± 27 ng/ml at PD0, slightly increased during early lactation until PD14, and then decreased to reach 762 ± 12 ng/ml at weaning. The concentration of TGF-β2 was much lower than that of TGF-β1 in mouse milk, around 105 ± 18 ng/ml at the beginning of lactation, decreased significantly during mid-lactation, and reached a minimum at PD14. At the end of lactation, the concentration of TGF-β2 was similar to that of early lactation, 89 ± 10 ng/ml. The concentration of IGF-1 was very low at the beginning of lactation, less than 100 ng/ml. It then increased by PD14, arrived at a maximum at PD18 (445 ± 83 ng/ml) and then decreased at weaning. The change in VEGF in mouse milk was different from that of other growth factors; it started at 55 ± 8 ng/ml at PD0 and then decreased to 35 ± 6 ng/ml at PD5. After PD10, the level of VEGF slightly changed to around 50 ng/ml and then further up to 63 ± 7 ng/ml at weaning (**Figure 3**). We also tried to measure the concentration of PDGF, but it was undetectable in mouse milk at all times measured (< 3.83 pg/ml).

Correlation analysis was conducted for EGF, IGF-1, TGF-β1 and 2, and VEGF milk levels with those of their receptor mRNA expression in mouse small intestine, which showed that TGF-β1 was significantly correlated with neonatal intestinal TGF-β1R (**Figure 4**).

DISCUSSION

The small intestine, as the major organ for nutrient absorption and the defense barrier for pathogens in infants, plays a crucial role in growth and health of the newborn. Therefore, maintenance of intestinal homeostasis is vitally important for providing nutrients and reducing neonatal morbidity and mortality (15). Breast milk provides nutrients in adequate amounts to support neonatal growth and to stimulate development of the immune system (16). Growth factors are important components of whey proteins that exert their functions through

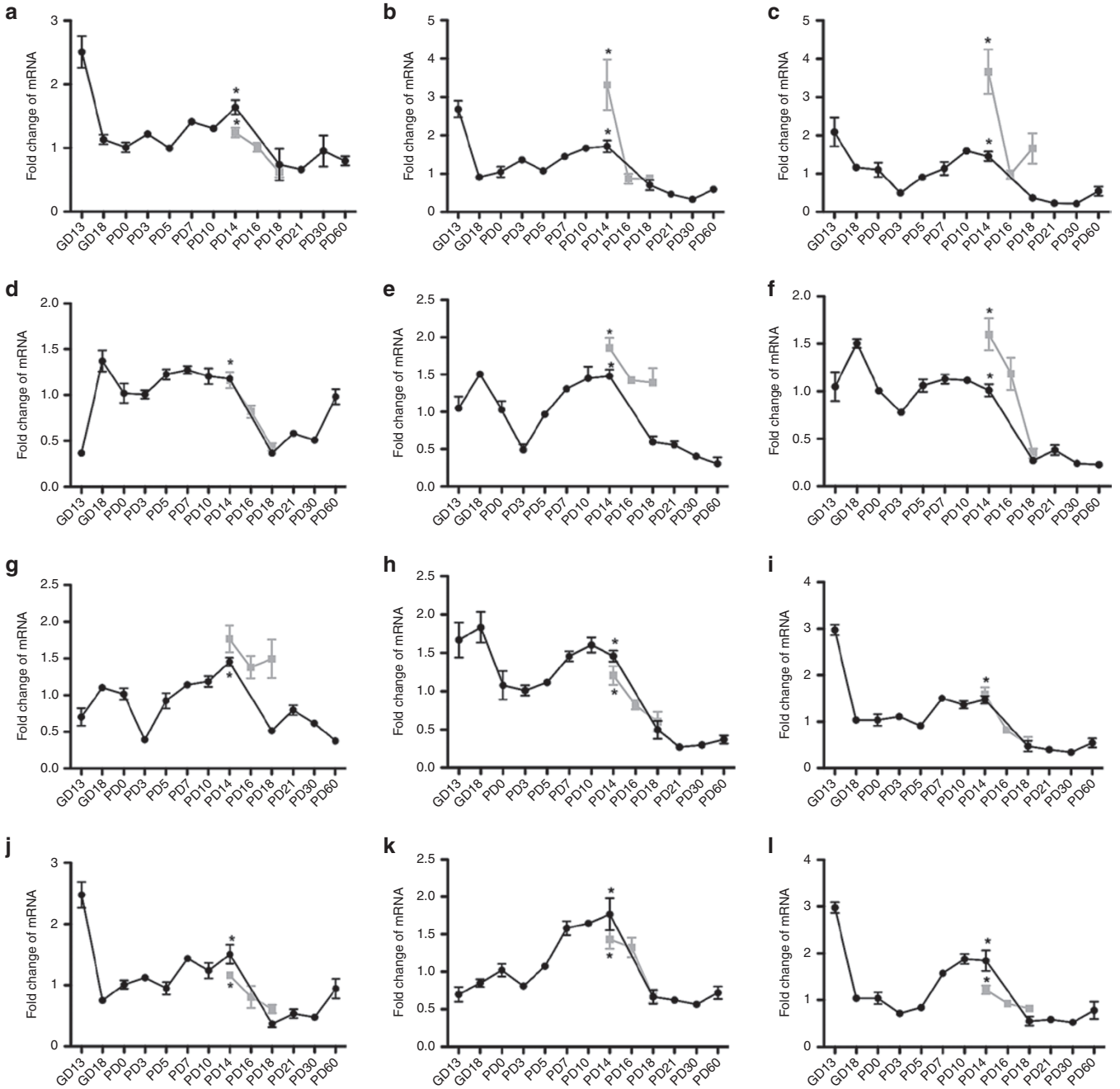

Figure 2. Relative growth factor receptor gene expression in mouse small intestine. Neonatal mouse small intestines were collected from normal intake (NI, closed circle) (gestational day (GD)13, GD18, PD0, PD3, PD5, PD7, PD10, PD14, PD18, PD21, PD30, PD60) and solid food restricted intake (RI, gray square) (postnatal day (PD)0, PD14, PD16, PD18) groups. Expression patterns of growth factor receptors were analyzed by real-time qPCR. In the RI group, solid food restriction was conducted by removing chow when pups were fed from PD 14 to PD 18. Samples from PD0 were used as a control. In both RI and NI groups, growth factor receptor expression at PD14 was significantly higher than that at PD18. (**a**) EGF receptor, (**b**) FGF receptor 1, (**c**) FGF receptor 2, (**d**) HGF receptor, (**e**) IGF1 and (**f**) IGF2 receptor, (**g**) Insulin receptor, (**h**) NGF receptor, (**i**) PDGF receptor, (**j**) TGF-β receptor 1, (**k**) TGF-β receptor 2, (**l**) VEGF receptor. Values are means ± SEM, $n = 3 \times (5–8)$ pups. * indicates $P < 0.05$.

binding to specific receptors. In this study, we used a mouse model to explore the paired expression of growth factors and of their respective small intestinal receptors.

We initially studied protein expression of growth factor receptors on the small intestinal epithelium. Variations in the expression of these proteins during infancy persisting to PD60 were found for all receptors analyzed. We also found that none of the four conventional internal controls, β-actin, GAPDH, β-tubulin, and total ERK1/2, were consistently expressed during infancy, which is possibly due to the fact that the perinatal period in mouse is brief and the rapid protein turnover during neonatal growth likely causes rapid changes in other functional molecules during that time. In our experiment, GAPDH mRNA was stably expressed in early infancy, and therefore

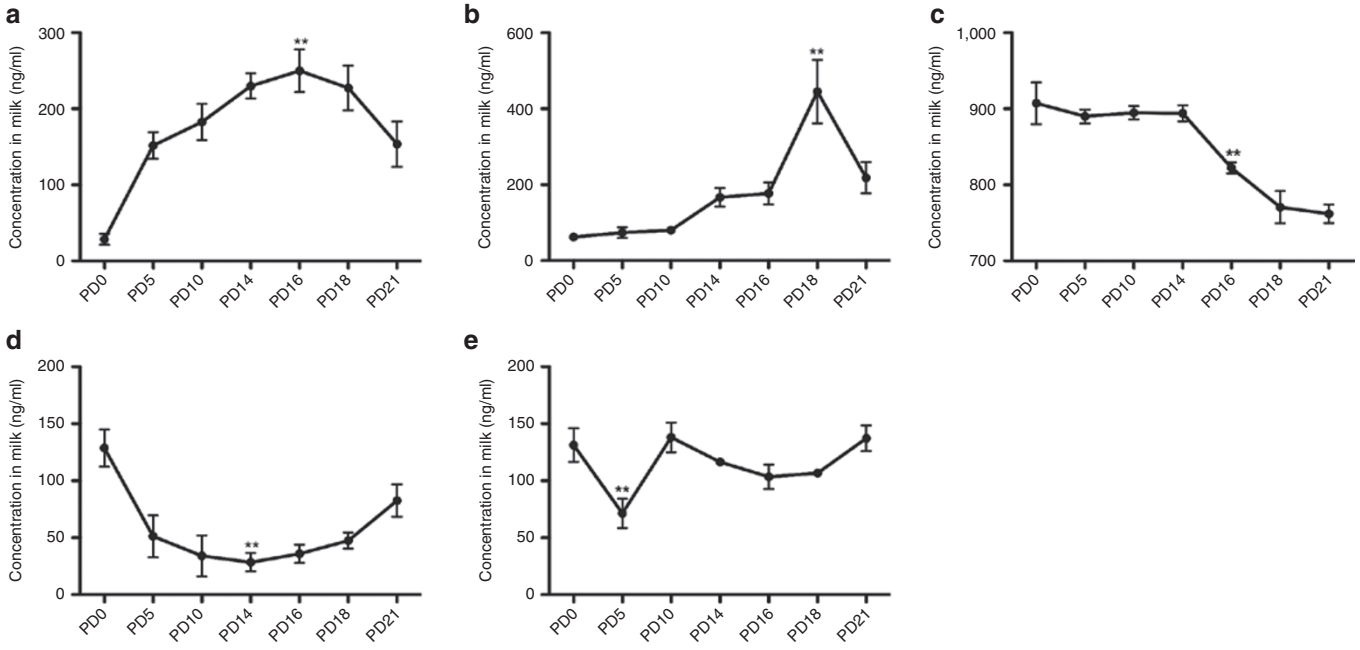

Figure 3. Growth factor concentration in mouse milk during lactation. Mouse milk was collected during the first 3 wk of lactation, and analyzed by enzyme-linked immunosorbent assay to detect levels of (**a**) EGF, (**b**) IGF-1, (**c**) TGF-β1, (**d**) TGF-β2, and (**e**) VEGF. Values are means ± SEM, $n = 6$. * indicates $P < 0.01$.

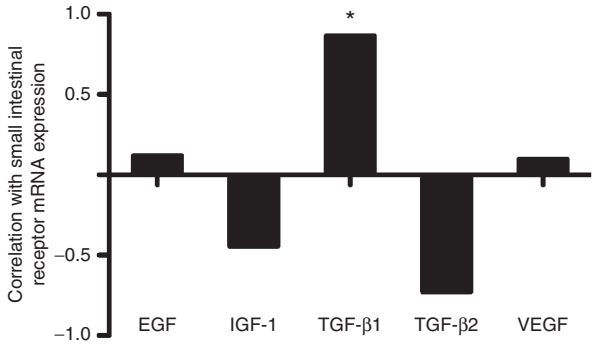

Figure 4. Pearson correlation analysis of milk growth factors (x-axis) and small intestinal growth factor receptor mRNA expression. Correlation was computed from all samples of PD0, PD5, PD10, PD14, PD18, PD21. * indicates $P < 0.05$.

growth factor receptor expression was analyzed at the mRNA level using GAPDH as an internal control.

In this study, gene expression of 12 growth factor receptors was analyzed. EGFR, FGFRs, HGFR, IGFRs, InsR, NGFR, PDGFR, TGF-βR2, and VEGFR showed maximum expression during the fetal period and then decreased at birth. The expression pattern for TGF-β2 was different and lower during fetal life compared with PD0. This may be explained by the fact that TGF-βR2 mRNA is restricted to the stroma and is not detected in the inner epithelial lining and smooth muscle layer of the gut at GD14 (17).

Starting from PD0, the expression of all growth factor receptors increased and arrived at a maximum by PD14. A sharp decrease in expression occurred from PD14 to PD18 for all receptors. The pattern for IGF-1R expression in piglets is consistent with our results, being higher in newborn animals and lower in weaning animals with a transient increase during the suckling period, although these authors did not specify the exact days (18). TGF-βR1 and TGF-βR2 have been shown to be located on the apical membrane of rat pup small intestine and their expression increased with age (19). A previous study using an EGF binding assay suggests that the binding capacity is more accentuated from day 14 onwards (20) in the Swiss ICR mouse. The difference between those results and our qPCR results may be due to the different strains used. Further, the increase in EGF binding capability may be associated with the decrease in its receptor expression at that time. One study reported that the level of FGFR3 reached a peak from postnatal day 7 to 21 and then decreased to a low level in adult FVB/N mice (21). There are very limited data on the expression patterns for receptors for HGF, NGF, VEGF, and PDGF, which stress the relevance of our current findings for the understanding of intestinal development.

In our study, the expression of all growth factor receptors analyzed was found to decrease sharply from PD14 to PD18, the third week of postnatal life, which is the stage when the neonatal small intestine dramatically changes in length, villus height, and enzyme expression and also the time for transition from milk to solid food, including chow, bedding, and feces (22). To avoid effects from solid food consumption, an experiment was conducted by removing pups at all times when dams were given solid food from PD14 to PD18. The results suggest similar trends in the absence or the presence of pup access to solid food. Receptors decreased greatly after PD14, even though expression of FGFR1, FGFR2, IGF-2R, and VEGFR at PD14 and that of IGF-1R and InsR at PD18 changed compared to the normally fed group, but this did not last.

This demonstrates that solid food intake does not affect the expression patterns for GF receptors although their expression changes somewhat at PD14 and PD18. These results suggest that adding complementary foods to the infant diet may not significantly alter neonatal intestinal maturation. The decrease in GF receptors from PD 14 to PD 18 may be involved in the closure process of the small intestine which in rodents occurs from PD 16 to PD 23 and may represent a redifferentiation of the small intestinal epithelium present from birth and adjusting for the changeover from suckling to the weaning period (23).

We hypothesized that the expression patterns for growth factor receptors may be correlated with the concentration of growth factors in milk. EGF, IGF-1, PDGF, TGF-β1 and 2, and VEGF were determined in mouse milk. The concentration of EGF started low in colostrum, increased and reached the maximum (250 ± 28 ng/ml) at PD16, and then decreased at weaning. Most studies on EGF in rodent milk have shown consistent results with an increase until day 15–19 of lactation (24–26) except for Grueters *et al.* who found a maximum level on day 6 of lactation (27). Different from its abundance in human colostrum, the maximum level of EGF in mouse milk occurred during mid-lactation. In our study, expression of EGFR in postnatal small intestine reached a maximum at PD14 and decreased at PD16 which may be due to the occurence of peak milk EGF on that day. Correlation analysis showed that there is no significant association between milk-born EGF and the expresssion of EGFR during lactation, and that solid food restriction reduced EGFR expression although not significantly. This demonstrates that intraluminal EGF has a partial effect on the expression of its intestinal receptor but not sufficient to alter the pattern. It is evident that other factors are involved in regulating this.

There is limited information on TGF-β1 and 2 in rodent milk. TGF-β2 was shown to reach a maximum at PD6 in rat milk (28). In our study, TGF-β1 and 2 were detected in mouse milk, but showed different patterns during lactation. The concentration of TGF-β1 was stable until PD14 and then decreased, whereas the concentration of TGF-β2 was at a maximum at birth, decreasing and reaching a minimum at PD14. Our study showed a higher TGF-β1 content in mouse milk, which is different from the dominance of TGF-β2 in human and rat milk (29). The correlation analysis of TGF-β1 and its receptor indicated they were significantly associated. Solid food restriction further supported the correlation analysis by not significantly affecting the expression of TGF-β1. The correlation between TGF-β2 and its receptor was negative but not significant. From previous studies, it is not clear why TGF-β1 and 2 would have a different response in mouse milk (28,29).

IGF-1 plays an important role in villus and crypt growth, nutrient absorption, and infant health (30,31). Milk IGF-1 levels started low at birth, arrived at a maximum at PD18 and then decreased toward weaning. The change in VEGF in mouse milk was different from that of other growth factors. There was no significant change in VEGF during lactation except for a sudden decrease at PD5. Although the role of VEGF in the small intestine is not clear, it is likely a biologically active factor due to its abundance in human colostrum (32). Furthermore, breast milk collected from preterm mothers has a higher concentration of VEGF than breast milk from term mothers (33). VEGF does not stimulate intestinal epithelial cell growth (34), but intestines from infants with NEC show decreased VEGF by immunohistochemistry (35). Our study is the first to report levels of IGF-1 and VEGF in mouse milk. From our results, it appears that levels of IGF-1 and VEGF during lactation are not correlated to the regulation of their respective receptors in the neonatal intestine.

Our results do not support that gene expression of growth factor receptors in neonatal intestine is exclusively affected by their corresponding milk growth factors, albeit one receptor, TGF-β1R, significantly correlates with the milk TGF-β1 concentration. Levels of endogenous growth factors in the offspring were not analyzed in this study, although as the infant small intestine matures, growth factors may be excreted. Endogenous production may also play a role in intestinal receptor expression. A study on rat duodenum found that endogenous TGF-β1 production is low at birth, but increases after mid-weaning (36). Immunohistochemical localization of TGF-α in porcine intestine showed that expression is greater at PD14 and 21 than in PD7 piglets (37). TGF-α belongs to the EGF family and binds to the EGF receptor (37). If TGF-α regulates EGF receptor expression significantly, its decrease by PD14 may be explained. Concentrations of growth factors may not be the critical factor in determining their functions, which are more dependent on the strength of signals from the cells to the nucleus (29). Besides endogenous growth factors, other intrinsic factors such as glucocorticoid may play a role in expression of growth factor receptors during infancy. The age for significant changes in growth factor receptors, PD14, coincides with the second surge, a marked increase of glucocorticoid in the small intestine (38). An effect of glucocorticoid on sustained EGFR activity was shown in a receptor knockout mouse model (39).

In summary, by using a mouse model, we analyzed the developmental pattern of 12 growth factor receptors in the neonatal small intestine, which mostly showed similar gene expression patterns. Paired data was obtained for five growth factors and their corresponding small intestinal receptors. Our study showed that the expression of growth factor receptors in mouse neonatal small intestine increases after birth and decreases significantly after PD14, which was not affected by introduction of solid food. Analyses of milk growth factors as well as the correlation analysis during lactation suggest that gene expression of growth factor receptors in the small intestine is likely orchestrated by multiple factors other than milk growth factors content alone.

METHODS

Sample Collection

C57BL/6N male (n = 14) and female mice (n = 30) were purchased from Charles River Laboratory (Wilmington, MA) and fed standard chow diet (LabDiet 5001, Purina, Hayward, CA). Mice were housed in solid plastic hanging cages with a 12 h dark–light cycle under constant

conditions. Mice were bred overnight and the appearance of a vaginal plug determined successful breeding with the day counted as gestational day (GD) 0. Newborn pups (eight per dam) were counted as postnatal day (PD) 0 and were weaned at PD21 to the chow diet fed *ad libitum*. Samples of small intestine, including duodenum and jejunum, were collected at regular intervals from GD13 to PD60. Milk was collected at PD0, 5, 10, 14, 16, 18, and 21. All animal procedures were approved by the University of California, Davis Institutional Animal Care and Use Committee.

Solid Food Restriction

Solid food was removed from the dam between PD14 to PD18 when mouse pups were allowed to nurse. Every 6 h, the dam was moved to a different cage with food for 1 h, and put back to the original cage to continue feeding pups. Small intestine samples were collected at PD14, PD16, and PD18.

Membrane Protein Extraction

Small intestines were collected at the designated ages and perfused by 1× phosphate-buffered saline (PBS). The Mem-PER Plus kit (Thermo Scientific, Rockford, IL) protocol was then carried out to obtain the membrane proteins.

Immunoblotting Analysis

Membrane proteins (50 µg) were electrophoresed through 10% polyacrylamide gel, transferred onto nitrocellulose membrane at 350 mA for 60 min, and blocked for 1 h in 1× PBS/0.1% Tween-20 (v/v) (PBST) with 5% (w/v) non-fat milk at 4 °C. Antibodies against EGF receptor (Santa Cruz Biotechnologies, Paso Robles, CA), TGF-β1&2 receptors (Thermo Scientific, Rockford, IL), IGF-1 receptor (Cell Signaling, Danvers, MA), and insulin receptor (Cell Signaling) were diluted 500 times in 5% BSA and incubated with the membrane overnight. After washing, membranes were incubated with the corresponding secondary antibody (1:5,000 in 5% non-fat milk) for 1 h at room temperature. After washing three times, the signal was detected using the ECL plus reagent (Pierce, Waltham, MA). All data were normalized to ERK1/2 protein levels.

Real-Time qPCR

Total RNA from mouse small intestines was isolated using TRIzol reagent (Life Technologies, Carlsbad, CA) at regular intervals from GD 13 to PD 60. cDNA synthesis was conducted by the High Capacity of RNA to cDNA kit from Applied Biosystems (Waltham, MA). The real-time qPCR reaction was performed using the following protocol: 95 °C for 10 min; 40 cycles of 95 °C for 15 s, 60°C for 30 s. Expression of target genes was analyzed and normalized to GAPDH (n = 6). Primers are provided in **Supplementary Table S1** online.

Growth Factors in Milk

Mouse milk was collected at PD0, PD5, PD10, PD14, PD16, PD18, and PD21, and centrifuged at 16,000 × g for 20 min at 4 °C to remove fat and casein. Samples were kept frozen at −80 °C until further analyses. EGF, TGF-β1&2, IGF-1, and VEGF concentrations in the whey were determined using enzyme-linked immunosorbent assay kits from R&D (Minneapolis, MN).

Statistics

All data were analyzed by Prism GraphPad Software (Version 5.0c, La Jolla, CA). Statistical analysis was performed by one-way ANOVA. Differences were considered significant when $P < 0.05$.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/pr

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