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Fetal Bone Formation Is Decreased from Middle Pregnancy to Birth

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Fetal bone development is a complex process that is regulated and maintained by minerals, hormones, and growth factors delivered from the mother via the placenta. Various biochemical markers of fetal bone development have been identified. However, many aspects of this process remain unclear. The aim of the study was to measure the activities of serum tartrate-resistant acid phosphatase type 5b (TRACP 5b) as a bone resorption marker and bone alkaline phosphatase (BAP) as a bone formation marker in preterm and term neonates, and to investigate fetal bone development in middle and late pregnancy. The study included 111 neonates (87 preterm and 24 term) born at Dokkyo Medical University Hospital. Neonates with illnesses and maternal diseases were excluded. Serum samples were collected within 3 hours after birth and stored at −80°C. Univariate and multivariate linear regression analyses were performed. The 111 neonates (median birth weight, 1,510 g) were born at a median of 31.3 weeks of gestation, and had TRACP 5b and BAP activities of 10.9 ± 4.0 U/L and 127.5 ± 49.2 U/L, respectively. TRACP 5b activity showed a tendency to be higher in term neonates, while BAP activity tended to be lower in term neonates. Importantly, TRACP 5b activity was positively correlated with gestational age and birth weight, and BAP activity was negatively correlated with gestational age, rate of born small-for-gestational-age neonates, and birth weight. These results suggest that bone formation during fetal growth is gradually decreased from middle pregnancy to birth, whereas bone resorption is gradually increased.

Keywords: bone alkaline phosphatase; fetal bone development; preterm neonate; tartrate-resistant acid phosphatase type 5b; term neonate
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Introduction

Fetal bone development is a complex process that is regulated and maintained by minerals, hormones, and growth factors delivered from the mother via the placenta (Itani and Tsang 2006; Mitchell and Jüppner 2010). Minerals required for fetal bone development are mostly transferred to the fetus in late pregnancy; however, many aspects of this process remain unclear (Shaw 1973; Ziegler et al. 1976). Various biochemical markers of bone development have been identified in cord blood and blood of neonates (Itani and Tsang 2006); and in Japanese adults, bone resorption markers in urine are commonly used to assess bone metabolism. However, this has the disadvantages that samples need to be collected at the same time for each test to account for circadian rhythm, and results require correction using urinary creatinine levels due to individual differences in renal clearance (Nishizawa et al. 2008).

Serum tartrate-resistant acid phosphatase type 5b (TRACP 5b) is a biochemical marker of bone metabolism that is mainly derived from osteoclasts. TRACP 5b activity is used clinically as a bone resorption marker in adults (Shidara et al. 2008; Yamada et al. 2008), and normal TRACP 5b activities are 1.7-5.9 U/L in men, 1.2-4.4 U/L in premenopausal women, and 2.5-7.6 U/L in postmenopausal women in Japan (Nishizawa et al. 2008). The normal serum TRACP 5b activity in neonates has not been determined. Serum total alkaline phosphatase (ALP) is a nonspecific indicator of osteoblast activity, and therefore, bone alkaline phosphatase (BAP) can be used to assess bone formation. BAP levels are reliable markers of bone formation in the perinatal period (Uemura et al. 2002).

The aim of this study was to measure serum TRACP 5b activity immediately after birth in term and preterm neo-

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nates to determine reference ranges, and to investigate fetal bone development in pregnancy using TRACP 5b activity as a bone resorption marker and the BAP activity as a bone formation marker.

Subjects and Methods

Subjects and samples

The subjects were 111 neonates (87 preterm and 24 term) born at 25-41 weeks of gestation who were born at Dokkyo University Hospital between February 2005 and March 2006. Neonates with metabolic bone diseases, kidney and digestive tract diseases, severe congenital anomalies, and maternal endocrine diseases were excluded. Written informed consent was obtained from parents for enrollment in the study. Serum samples from neonates were collected via arterial blood sampling or venipuncture or capillary blood from a heel lance within 3 h after birth and stored at −80°C until measurement of TRACP 5b and BAP. The study was approved by the ethics committee of Dokkyo Medical University Hospital.

Measurement of TRACP 5b and BAP

TRACP 5b activity was measured with an Osteolinks TRAP-5b kit (Approval of manufacturing/marketing of medical devices: 22000AMX00076000. DS Pharma Biomedical Corp., Osaka, Japan). Prior to introduction of this kit, measurement of osteoclast-derived TRACP 5b activity was difficult because of interference by acid phosphatase released into the serum by red blood cells and macrophage-derived TRACP type 5a. However, the Osteolinks TRAP-5b kit enables accurate measurement of the TRACP 5b level using a fragment-absorbed immunocapture enzymatic assay (FAICEA). This method uses two monoclonal antibodies (anti-active TRACP 5b antibody and anti-inactive TRACP 5b antibody) and is based on the optimum pH of TRACP type 5a differing from that of TRACP 5b (Halleen et al. 2000; Igarashi et al. 2001; Ohashi et al. 2007). BAP was measured with an Osteolinks BAP kit (Approval of manufacturing/marketing of medical devices: 21000AMY00180000. DS Pharma Biomedical Corp.) using an immunoassay in a microtiter strip format with a monoclonal anti-BAP antibody coated on the strip to capture BAP in the sample. The enzyme activity of the captured BAP is detected with a p-nitrophenyl phosphate substrate (Gomez et al. 1995).

Statistical analysis

Continuous data are presented as mean ± standard deviation (SD) (normally distributed data) or median and interquartile range (IQR) [25%, 75%] (not normally distributed data). Categorical data are presented as number (percentage). Univariate and multivariate linear regression analyses were performed to determine whether TRACP 5b and BAP activities were associated with gestational age, sex, birth weight, and born small-for-gestational-age (SGA) neonates (birth weight < 10^{th} percentile from the mean). Covariates with high collinearity (Spearman rank correlation coefficient [ρ] > 0.8) were not simultaneously included in the analysis. When building multivariate models, birth weight (model 1) or gestational age (model 2) were excluded to avoid multicollinearity among covariates. All analyses were performed using SPSS ver. 22.0 for Windows (IBM, Armonk, NY). A P-value < 0.05 was considered significant.

Results

The characteristics of the neonates are shown in Table 1. A total of 354 neonates were admitted to Dokkyo University hospital during the study period. Of 123 neonates for whom written consent to inclusion in the study was obtained from both parents, 12 were excluded for the following reasons: 1 had a mother with epilepsy, 1 had a mother with scleroderma, 1 had thanatophoric dysplasia, 3 were a set of triplets, and 6 were in 3 sets of twins. Therefore, 111 neonates (57 males, 54 females; 28 SGA; 87 preterm, 24 term) were included in the study. These neonates were born at a median of 31.3 (IQR = 28.9-33.9) weeks of gestation with a median birth weight of 1,510 (IQR = 980-2,258) g. The mean TRACP 5b and BAP activities were 10.9 ± 4.0 U/L and 127.5 ± 49.2 U/L, respectively. In the 87 preterm and 24 term neonates, TRACP 5b activities were 10.4 ± 4.1 and 12.6 ± 3.1 U/L and BAP activities were 131.1 ± 52.3 and 114.4 ± 33.6 U/L, respectively. Neither comparison was significant, but TRACP 5b activity tended to be higher in term neonates and BAP activity tended to be higher in preterm neonates.

In univariate linear regression analysis, TRACP 5b activity was significantly associated with gestational age and birth weight (Table 2). Scatter plots of correlations of TRACP 5b activity with gestational age and birth weight are shown in Fig. 1. Gestational age was also strongly correlated with birth weight (Spearman $\rho = 0.912$, $P < 0.001$). Therefore, when building multivariate models, birth weight (model 1) or gestational age (model 2) was excluded to avoid multicollinearity among covariates. In multivariate linear regression analysis, TRACP 5b activity was significantly positively correlated with gestational age in model 1 (regression coefficient [B] = 0.041, standardized regression coefficient [β] = 0.343, $P < 0.001$; adjusted R^2 [model 1] = 0.097) and birth weight in model 2 ($B = 0.001$, $\beta = 0.297$, $P = 0.004$; adjusted R^2 [model 2] = 0.057).

In univariate linear regression analysis, BAP activities were not significantly associated with any variable (Table 3). Scatter plots of BAP activities with gestational age and birth weight are shown in Fig. 2. In multivariate linear regression analysis, BAP activities were significantly negatively correlated with gestational age and rate of born SGA neonates in model 1 (gestational age: $B = -0.306$, $\beta = -0.208$, $P = 0.031$; SGA neonates: $B = -24.410$, $\beta = -0.214$, $P = 0.026$; adjusted R^2 [model 1] = 0.049), and birth weight and born SGA neonates in model 2 (birth weight: $B = -0.012$, $\beta = -0.211$, $P = 0.040$; SGA neonates: $B = -29.852$, $\beta = -0.261$, $P = 0.011$; adjusted R^2 [model 2] = 0.045).

Scatter plots of TRACP 5b and BAP activities with gestational age in males and females indicated no differences between the sexes (Fig. 3). Scatter plots of TRACP 5b and BAP activities with gestational age in SGA and appropriate-for-gestational age (AGA) neonates also indicated no difference between these types of neonates (Fig. 4).

Table 1. Characteristics of the neonates (n = 111).

Item	Data
Number	111
Gender (male/female)	57/54 (51.4%/48.6%)
Gestational age (weeks)	31.3 [28.9–33.9]
Birth weight (g)	1,510 [980–2,258]
TRACP 5b activity (U/L)	10.9 ± 4.0
BAP activity (U/L)	127.5 ± 49.2
SGA neonates (Yes/No)	28/83 (25.2%/74.8%)

Continuous data are presented as mean ± SD (normally distributed data) or as median and interquartile range (IQR) (not normally distributed data).
Categorical data are presented as number (percentage).
TRACP 5b, tartrate-resistant acid phosphatase type 5b; BAP, bone alkaline phosphatase; SGA, small-for-gestational-age.

Table 2. Linear regression analysis for TRACP 5b activity (U/L) (n = 111).

	TRACP 5b			
	B	SE	*β*	*P*-value
Univariate model				
Male (vs. female)	-0.657	0.767	-0.082	0.393
Gestational age (per week)	0.393	0.011	0.343	**< 0.001**
Birth weight (per g)	0.001	0.000	0.268	**0.004**
SGA neonates, yes (vs. no)	-0.263	0.877	-0.029	0.765
Multivariate model 1				
Male (vs. female)	-0.337	0.733	-0.042	0.646
Gestational age (per week)	0.041	0.011	0.343	**< 0.001**
Birth weight (per g)	-			
SGA neonates, yes (vs. no)	0.172	0.855	0.019	0.841
Multivariate model 2				
Male (vs. female)	-0.468	0.746	-0.059	0.532
Gestational age (per week)	-			
Birth weight (per g)	0.001	0.000	0.297	**0.004**
SGA neonates, yes (vs. no)	0.721	0.933	0.078	0.441

Multivariate model 1: excludes birth weight. Adjusted $R^2 = 0.097$.
Multivariate model 2: excludes gestational age. Adjusted $R^2 = 0.057$.
TRACP 5b, tartrate-resistant acid phosphatase type 5b; SGA, small-for-gestational-age; *B*, regression coefficient; SE, standard error of the regression coefficient; *β*, standardized regression coefficient.

Discussion

Advances in perinatal care, including pulmonary surfactant replacement therapy, high-frequency oscillatory ventilation, prenatal administration of glucocorticosteroids, regionalization, and prenatal maternal transfer, have reduced mortality in preterm neonates (Itabashi et al. 2009). Despite these advances, there is still a poor understanding of fetal bone development, but it is known that a mechanism to maintain rapid bone development is established in late pregnancy, in which large amounts of calcium and phosphorus from the mother are deposited in fetal bone, while bone resorption is inhibited (Shaw 1973; Ziegler et al. 1976; Itani and Tsang 2006; Mitchell and Jüppner 2010).

In this study, the mean TRACP 5b and BAP activities were 10.9 ± 4.0 U/L and 127.5 ± 49.2 U/L, respectively, in neonates, with tendencies for a higher TRACP 5b activity in term neonates and a higher BAP activity in preterm neonates. TRACP 5b activity was positively correlated with gestational age and birth weight, and BAP activities were negatively correlated with gestational age, born SGA neonates, and birth weight. Few studies have examined TRACP 5b levels in infancy, but in a study of 404 children, Chen et al. (2005) found that TRACP 5b activity was high in the neonatal period, slowly decreased after infancy, and then increased at 12-13 years in boys and at 10-11 years in girls.

The mean TRACP 5b activities in cord blood of 28 neonates in Chen et al. (2005) were 5.0 ± 0.8 U/L in males and 5.9 ± 1.0 U/L in females, with significantly higher lev-

Table 3. Linear regression analysis for BAP activity (U/L) (n = 111).

	BAP			
	B	SE	*β*	*P*-value
Univariate model				
Male (vs. female)	2.211	9.463	0.022	0.816
Gestational age (per week)	-0.259	0.139	-0.176	0.065
Birth weight (per g)	-0.007	0.006	-0.114	0.232
SGA neonates, yes (vs. no)	-19.564	10.633	-0.174	0.068
Multivariate model 1				
Male (vs. female)	-0.163	9.254	-0.002	0.986
Gestational age (per week)	-0.306	0.140	-0.208	**0.031**
Birth weight (per g)	-			
SGA neonates, yes (vs. no)	-24.410	10.801	-0.214	**0.026**
Multivariate model 2				
Male (vs. female)	0.564	9.242	0.006	0.951
Gestational age (per week)	-			
Birth weight (per g)	-0.012	0.006	-0.211	**0.040**
SGA neonates, yes (vs. no)	-29.852	11.556	-0.261	**0.011**

Multivariate model 1: excludes birth weight. Adjusted $R^2 = 0.049$.
Multivariate model 2: excludes gestational age. Adjusted $R^2 = 0.045$.
BAP, bone alkaline phosphatase; SGA, small-for-gestational-age; *B*, regression coefficient; SE, standard error of the regression coefficient; *β*, standardized regression coefficient.

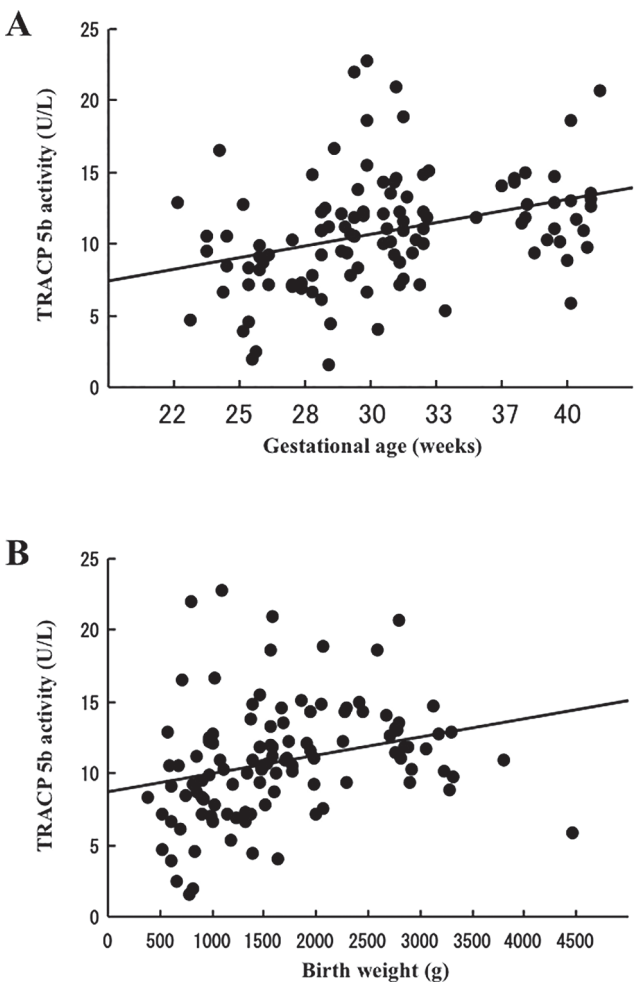

Fig. 1. Relationships of TRACP 5b activity with gestational age and birth weight.
Scatter plots of TRACP 5b activity (U/L) with gestational age (weeks) [A] and birth weight (g) [B] (n = 111 for each graph).

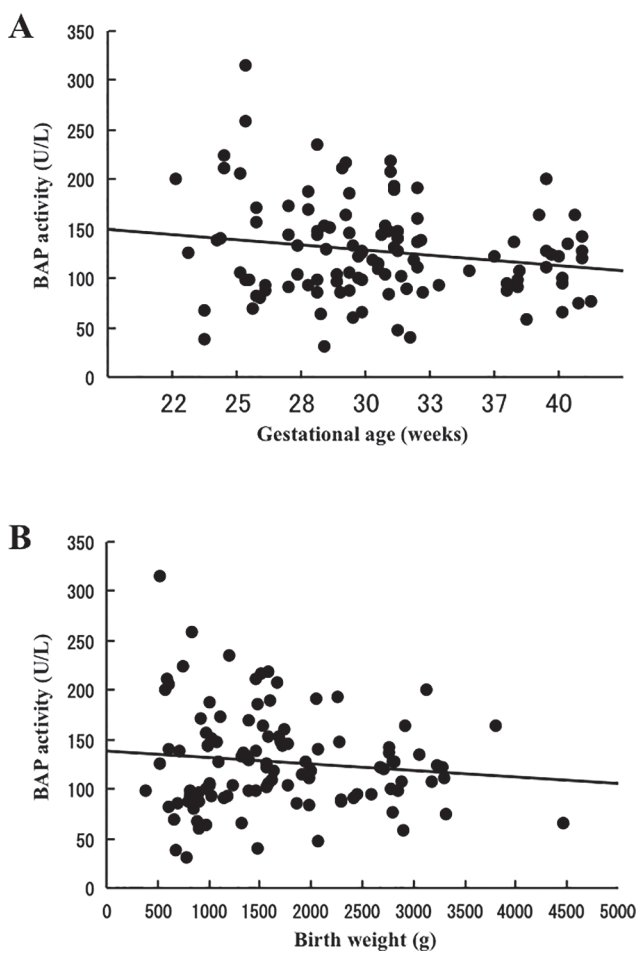

Fig. 2. BAP activity, gestational age and birth weight.
Scatter plots of BAP activity (U/L) with gestational age (weeks) [A] and birth weight (g) [B] (n = 111 for each graph).

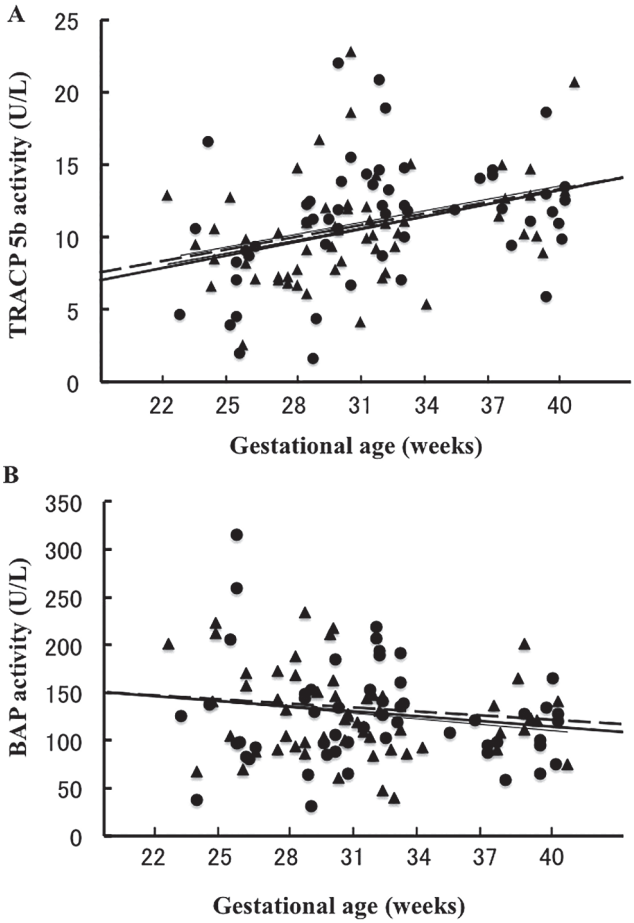

Fig. 3. Relationships of TRACP 5b and BAP activities with gestational age in different sexes.
Scatter plots of TRACP 5b activity (U/L) with gestational age (weeks) [A] and BAP activity (U/L) with gestational age (weeks) [B] in males (n = 57: ▲) and females (n = 54: ●). Solid and dotted lines show approximate lines for males and females, respectively.

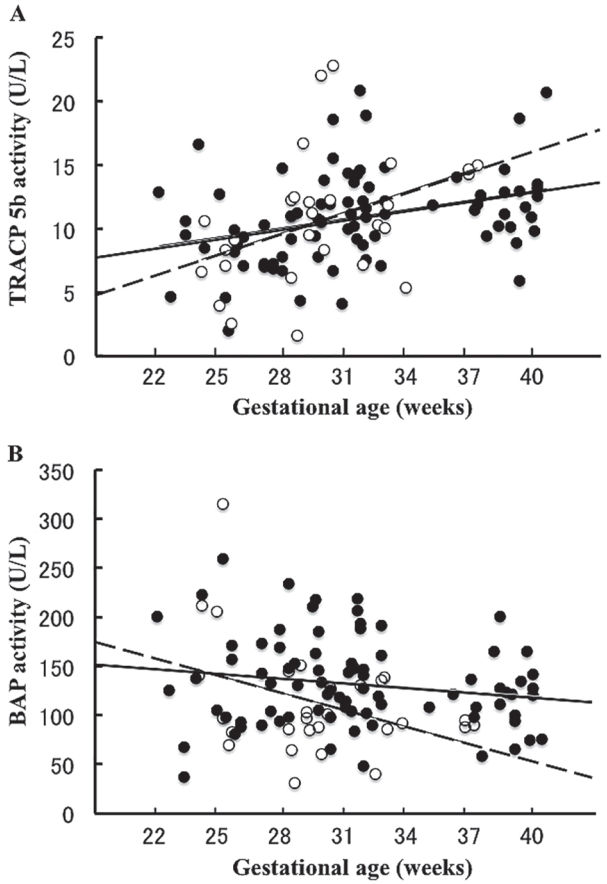

Fig. 4. Relationships of TRACP 5b and BAP activities with gestational age in AGA and SGA neonates.
Scatter plots of TRACP 5b activity (U/L) with gestational age (weeks) [A] and BAP activity (U/L) with gestational age (weeks) [B] in AGA (n =83: ●) and SGA (n = 28: ○) neonates. Solid and dotted lines show approximate lines for AGA and SGA neonates, respectively.

els in females. In contrast, there was no association between sex and TRACP 5b activity in the current study, consistent with the suggestion by Kovacs (2014) that testosterone and estradiol may not be important for fetal mineral homeostasis and skeletal development. In a study of bone metabolism markers in children aged 2 months to 18 years, Rauchenzauner et al. (2007) found higher TRACP 5b activity in younger children than in older children; however, no neonatal data were included. Fischer et al. (2012) measured bone metabolism markers in 424 healthy children aged 1 month to 21 years, and found that TRACP 5b and BAP activities were strongly correlated with each other; were significantly correlated with age, with the highest levels occurring during infancy and adolescence; and were significantly higher in boys than in girls during adolescence.

Alterations of TRACP 5b and BAP activities were found in the current study, but not in the neonatal period. In studies of other bone resorption markers in preterm neonates, Tsukahara et al. (1999) found an inverse correlation between urinary collagen crosslinks and gestational age in preterm newborns, and Nakano et al. (2006) found an inverse correlation between type 1 collagen C-terminal telopeptide (1CTP) in cord blood and gestational age in newborns at 27-42 weeks of gestation. These results differ from our findings. This may be due to fluctuation in serum 1CTP, which rises when renal function declines with prematurity of the kidney and is high in pycnodysostosis, which is caused by cathepsin K deficiency (Nishi et al. 1999). The difference in methods used for measuring TRACP 5b activity and collagen crosslinks may also account for the different results for these bone resorption markers. Nishizawa et al. (2008) have concluded that TRACP 5b activity measured in healthy Japanese subjects using a FAICEA is appropriate for evaluation of systemic osteoclastic activity. Therefore, TRACP 5b activity appears to be a more useful bone developmental marker than collagen crosslinks for evaluation of bone resorption.

Few studies have assessed biochemical markers in SGA neonates. Namgung et al. (1996) found no differences in 1CTP levels between 19 term SGA neonates and 38 term AGA neonates, similar to our findings for TRACP 5b activ-

ity. Briana et al. (2008) found that levels of BAP, ALP, osteocalcin (OC), and cross-linked *N*-telopeptide of type 1 collagen (NTx) did not differ significantly between 20 term SGA and 20 term AGA neonates. These findings differ from our results for BAP in SGA neonates, which may be because most of our SGA neonates were preterm (25/28). To the best of our knowledge, it is unknown how BAP levels fluctuate in preterm SGA neonates. Itabashi et al. (2007) assessed anthropometric measurements in 449 preterm SGA neonates and concluded that the overall length/height catch-up rates were 68% at 1 year, 89% at 3 years, and 88% at 5 years. This suggests that bone metabolism in SGA neonates may differ from that in AGA neonates in the fetal period. An analysis of a larger number of preterm SGA neonates is needed to examine this issue.

In this study, BAP activities were 3- to 10-fold higher in neonates than in adults and fetal bone formation was activated early in middle pregnancy. TRACP 5b activity was positively correlated with gestational age and birth weight, showing that fetal bone resorption is gradually activated in middle pregnancy. We have also found that TRACP 5b activity tends to continue to increase after birth until about 3 weeks of age, and remains constant thereafter (unpublished data). Further studies on TRACP 5b activity are needed to validate these results.

There are some limitations in the study. Bone mineral density and serum calcium and phosphorus levels were not evaluated, and chronological data were lacking for TRACP 5b activity after birth. Given the limited collection of data from only a few healthy neonates, the results may not appropriately define the normal ranges in healthy neonates.

In conclusion, TRACP 5b activity was positively correlated with gestational age and birth weight, and BAP activity was negatively correlated with gestational age, born SGA neonates, and birth weight. These results suggest that bone formation during fetal growth is gradually inactivated from middle pregnancy to birth, whereas bone resorption is gradually activated.

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Conflict of Interest

The authors declare no conflict of interest.

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