Table 1. *IFT122* mutation and their pathogenicity prediction

	Allele 1					Allele 2				
Family; Ref.	Nucleotide change	Amino acid change	SIFT	PolyPhen-2	MutationTaster	Nucleotide change	Amino acid change	SIFT	PolyPhen-2	MutationTaster
CED-01; Walczak-Sztulpa et al. (1)	c.1658T>G	p.V553G	0.00; damaging	1.000; probably damaging	1.000; disease causing	c.1658T>G	p.V553G	0.00; damaging	1.000; probably damaging	1.000; disease causing
CED-02; Walczak-Sztulpa et al. (1)	c.1118C>T	p.S373F	0.00; damaging	1.000; probably damaging	1.000; disease causing	c.1118C>T	p.S373F	0.00; damaging	1.000; probably damaging	1.000; disease causing
CED-03; Walczak-Sztulpa et al. (1)	c.21G>C	p.W7C	0.00; damaging	1.000; probably damaging	1.000; disease causing	c.502+5G>A	Splice-site change	–	–	–
This study	c.1636G>A	p.G546R	0.00; damaging	1.000; probably damaging	1.000; disease causing	c.1108delG	p.E370Sfs*51	–	–	–

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References

1. Walczak-Sztulpa J, Eggenschwiler J, Osborn D et al. Cranioectodermal dysplasia, sensenbrenner syndrome, is a ciliopathy caused by mutations in the IFT122 gene. Am J Hum Genet 2010: 86: 949–956.
2. Saitsu H, Nishimura T, Muramatsu K et al. De novo mutations in the autophagy gene WDR45 cause static encephalopathy of childhood with neurodegeneration in adulthood. Nat Genet 2013: 45: 445–449.
3. Eleftheriades M, Iavazzo C, Manolakos E et al. Recurrent short rib polydactyly syndrome. J Obstet Gynaecol 2013: 33: 14–16.
4. Cortellino S, Wang C, Wang B et al. Defective ciliogenesis, embryonic lethality and severe impairment of the Sonic Hedgehog pathway caused by inactivation of the mouse complex A intraflagellar transport gene Ift122/Wdr10, partially overlapping with the DNA repair gene Med1/Mbd4. Dev Biol 2009: 325: 225–237.
5. Bhuiyan ZA, Momenah TS, Gong Q et al. Recurrent intrauterine fetal loss due to near absence of HERG: clinical and functional characterization of a homozygous nonsense HERG Q1070X mutation. Heart Rhythm 2008: 5: 553–561.

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Multiple pregnancy, short cervix, part-time worker, steroid use, low educational level and male fetus are risk factors for preterm birth in Japan: A multicenter, prospective study

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Abstract

Aim: To examine the relationship between preterm birth and socioeconomic factors, past history, cervical length, cervical interleukin-8, bacterial vaginosis, underlying diseases, use of medication, employment status, sex of the fetus and multiple pregnancy.

Methods: In a multicenter, prospective, observational study, 1810 Japanese women registering their future delivery were enrolled at 8^{+0} to 12^{+6} weeks of gestation. Data on cervical length and delivery were obtained from 1365 pregnant women. Multivariate logistic regression analysis was performed.

Results: Short cervical length, steroid use, multiple pregnancy and male fetus were risk factors for preterm birth before 34 weeks of gestation. Multiple pregnancy, low educational level, short cervical length and part-timer were risk factors for preterm birth before 37 weeks of gestation.

Conclusion: Multiple pregnancy and cervical shortening at 20–24 weeks of gestation was a stronger risk factor for preterm birth. Any pregnant woman being part-time employee or low educational level, having a male fetus and requiring steroid treatment should be watched for the development of preterm birth.

Key words: cervical length, fetal sex, multiple pregnancy, part-time worker, steroid use.

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Introduction

Preterm birth (PTB), defined as delivery before 37 weeks of gestation, is a major cause of neonatal morbidity and mortality worldwide.[1,2] The Ministry of Health, Labor and Welfare in Japan reported that the incidence of PTB has increased in the last two decades. In 2010, preterm newborn infants represented 5.7% of live births. Several risk factors for PTB have been reported in many countries.[3–14] However, genetic background, environmental factors, and medical systems or quality could affect the risk differently in each country.

Clarifying the natural history of PTB and its associated risk factors in Japanese women will guide maternal–infant health-care professionals in applying a preventive strategy to reduce PTB. However, there has been no multicenter, prospective, observational study undertaken in Japan to clarify the risk factors for PTB. The objective of this study is to examine the prevalence of spontaneous PTB and determine the risk factors for PTB.

Methods

Study design and participants

A multicenter study was implemented in 14 reference obstetric units throughout Japan under the coordination of the Department of Obstetrics and Gynecology, University of Toyama, Toyama, Japan. The study was conducted from April 2008 to March 2010 and was approved by the institutional review board of each participating hospital or center. Participants provided written informed consent to study coordinators or investigators prior to participation in this study.

Women between 8^{+0} and 12^{+6} weeks of gestation were eligible for this study. During a regular prenatal visit, gestational age was based on the last menstrual period and the crown–rump length (CRL) around 9 weeks of gestation using transvaginal ultrasonography. If not, the CRL at approximately 9 weeks or the biparietal diameter after 12 weeks was used to define gestational age.

Data collection included maternal age, family income per year, educational level, previous pregnancy history (spontaneous miscarriage, induced abortion, spontaneous PTB, induced PTB), medical complications, underlying disease, smoking and alcohol intake habits, occupational status (full-time worker, part-time worker, full-time housewife), usage of medication (steroid for collagen disease or bronchial asthma, antihypertensive), Gram staining of cervical mucus, cervicovaginal interleukin-8 (IL-8), cervical length by vaginal ultrasonography at 20–24 weeks and sex of the fetus (Table 1).

Table 1 Risk factors for preterm birth examined in this study

Education
Family income
Obstetric history (e.g. spontaneous abortion, spontaneous preterm birth, stillbirth, fetal growth restriction, pregnancy-induced hypertension)
Infections (bacterial vaginosis, *Chlamydia trachomatis*, *Candida*)
Cigarette smoking and alcohol intake before and during pregnancy
Medications (steroids only for collagen disease or bronchial asthma, antihypertensives, anti-asthmatics and antidepressants)
Employment status (housewives, full-time worker, part-time worker)
Medical history or underlying disease (central nervous system, asthma, renal disease, heart disease, thyroid disease, bone or muscle disease, uterine myoma, collagen disease, hypertension, diabetes mellitus, psychological disease)
Gram staining at 8–12 weeks (modified Nugent score by Verstraeren)
Cervical length at 20–24 weeks
Cervical interleukin-8
Complication and pregnancy outcome in this pregnancy
Sex of fetus (male : female)

The first part of this study was scheduled between 8^{+0} and 12^{+6} weeks of gestation, and the second part was between 20 and 24 weeks of gestation. Cervical cytology and IL-8 samplings were carried out in each part, whereas the measurement of cervical length was performed only in visits between 20 and 24 weeks. The samples for Gram staining and IL-8 were obtained from cervical discharge with a small cotton swab applied for 10 s before the performance of any other portion of the pelvic examination. The samples for Gram staining were transferred to the Mitsubishi Chemical Medience Corporation (Tokyo, Japan) and were classified by the method of Verstraelen *et al.*[15] The samples for IL-8 were placed in buffer, frozen at–80°C within 2 h of collection, transferred to the University of Toyama, and analyzed within a few weeks using the enzyme-linked immunoassay method.[16] The level of cervicovaginal IL-8 and the category after modified classification of Gram staining were not announced to 14 reference obstetric units. Bacterial vaginosis (BV) was diagnosed and treated at respective hospitals.

The cervical length was measured with a transvaginal real-time ultrasonographic probe with an empty

maternal bladder. The appropriate view for measurement was determined by finding a faint line of echodensity or echolucency between the external os and the internal os. Undue pressure on the cervical canal, which might have artificially created the impression of a longer cervix, was avoided by withdrawing the probe until the image blurred and then reapplying only sufficient pressure to restore the image. The cervix was measured along the line made by the interface or the mucosal surfaces, and calipers were placed at the notches made by the internal os and the external os. The cervical length measurement recorded was the shortest measurement that was sufficiently clearly displayed.[17–20]

From all the participating centers, 1810 women were initially enrolled in the study (Fig. 1). Because we could not collect detailed data on pregnancy outcome from 218 women and on cervical length from 227 women, we recruited a total of 1365 women between April 2008 and March 2010.

The distribution of enrolled women by study center was as follows: Juntendo Urayasu Hospital (23.5%, $n = 321$), Aiiku Hospital (21.0%, $n = 286$), Sendai Medical Center (11.2%, $n = 153$), Hirosaki National Hospital (10.8%, $n = 147$), Okayama Medical Center (9.5%, $n = 129$), Seichokai Fuchu Hospital (7.3%, $n = 100$), Toyama University Hospital (6.2%, $n = 85$), Kyushu Medical Center (4.2%, $n = 57$), Kure Medical Center (3.4%, $n = 46$), Hamamatsu University Hospital (2.2%, $n = 30$) and National Center for Global Health and Medicine (0.8%, $n = 11$).

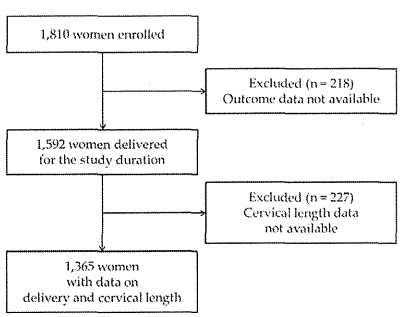

Figure 1 Participant flow diagram.

Statistical analysis

After univariate analysis, multivariate logistic regression analyses were employed to identify independent predictors of PTB. Odds ratios (OR) and 95% confidence intervals (95% CI) were reported with two-tailed probability (P) values. Statistical calculations were carried out using SAS ver. 17.1. A two-tailed P-value of 0.05 was used to define statistically significant results.

Results

Demographic and clinical characteristics

The majority of participants (99.5%) had completed high school. Approximately half of the women (49.8%) reported a high family income (≥¥5 million/year), 32.7% an intermediate income (¥2.00–4.99/year) and 1.9% a low income (<¥2 million/year). Twenty-four percent of women had experienced spontaneous abortion before 12 weeks. Although 30.0% of women quit smoking after their pregnancy was identified, 3.1% of women never quit smoking. Forty-two percent of women were housewives, while 14% of them worked part-time.

From the 1365 women, 42.3% presented with grade I microflora at 8–12 weeks, 1.4% showed an episode of grade I-like microflora, 23.1% showed an episode of heavy vaginal leukocytosis (grade I polymorphonuclear leukocytes [PMN]), while 21.3% of women had BV-like microflora.

The median maternal age was 32 years and the mean gestational age at delivery was 39 weeks. One hundred and two (7.5%) women experienced a spontaneous PTB before 37 weeks, while 19 (1.4%) did so before 34 weeks.

We have had six patients with steroid use (four patients for collagen disease, one patient for renal disease and one patient for bronchial asthma) and we included these six patients in multivariate logistic analysis. Two of six patients delivered preterm babies. To restrict the steroid users to the patients who had collagen disease or bronchial asthma, we counted the patients who had steroid for fetal lung maturity as non-steroid users.

Risk factors for PTB before 34 weeks of gestation

Table 2 provides details of the univariate analysis and the multivariate logistic regression analysis of risk factors for PTB before 34 weeks of gestation. In univariate analysis, low educational level, history of miscarriage or PTB, threatened abortion (atypical genital

Table 2 Risk factors for preterm birth before 34 weeks of gestation

		Univariate analysis			Multivariate analysis		
		OR	95% CI	*P*	OR	95% CI	*P*
Educational level							
1	Junior high school	26.29	2.60–265.75	0.0000**	10.20	0.50–208.09	0.13
2	Others						
Family income							
1	<¥2 million/year	3.1	0.40–24.24	0.26	—	—	—
2	Others						
Employment							
1	Part-time	1.23	0.35–4.23	0.75	—	—	—
2	Others						
History of miscarriage or preterm birth							
1	Yes	3.01	1.18–7.69	0.01547*	2.72	0.93–7.94	0.07
2	No						
History of cervical surgery (cone biopsy)							
1	Yes	N/A	N/A	N/A	—	—	—
2	No						
Uterine myoma							
1	Yes	2.54	0.57–11.30	0.20	—	—	—
2	No						
Threatened abortion							
1	Yes	3.67	1.19–11.37	0.0158*	1.75	0.44–7.02	0.43
2	No						
Subchorionic hematoma							
1	Yes	2.87	0.37–22.34	0.29	—	—	—
2	No						
Cervical length at 20–24 weeks <25 mm							
1	Yes	34.87	11.85–102.63	0.0000**	0.89†	0.84–0.94†	0.0000**
2	No						
Steroid use for collagen disease or bronchial asthma							
1	Yes	15.75	1.75–142.13	0.0097**	31.94	2.04–500.26	0.0136*
2	No						
Multiple pregnancy							
1	Yes	11.01	3.44–35.17	0.0000**	5.53	1.34–22.79	0.0179*
2	No						
Fetal sex							
1	Male	4.85	1.40–16.84	0.006**	5.06	1.35–19.00	0.0163*
2	Female						
Body mass index <18.5							
1	Yes	0.54	0.16–1.89	0.33	—	—	—
2	No						
Smoking during pregnancy							
1	Yes	N/A	N/A	N/A	—	—	—
2	No						
Smoking before pregnancy							
1	Yes	0.46	0.13–1.61	0.22	—	—	—
2	No						
Alcohol intake during pregnancy							
1	Yes	N/A	N/A	N/A	—	—	—
2	No						
Alcohol intake before pregnancy							
1	Yes	1.19	0.47–3.00	0.72	—	—	—
2	No						
Gram staining at 8–12 weeks: grade I PMN or BV-like							
1	Yes	0.94	0.37–2.37	0.89	—	—	—
2	No						
Chlamydial infection							
1	Yes	N/A	N/A	N/A			
2	No						
Candida infection							
1	Yes	1.12	0.15–8.55	0.91			
2	No						
IL-8 level at 20–24 weeks >360 ng/mL							
1	Yes	0.87	0.27–2.80	0.82	—	—	—
2	No						

**$P < 0.01$; *$P < 0.05$. †OR (95% CI) according to mm of cervical length. BV, bacterial vaginosis; CI, confidence interval; IL, interleukin; N/A, no one had premature delivery; OR, odds ratio; PMN, polymorphonuclear leukocytes.

bleeding at early pregnancy), steroid usage, multiple pregnancy, male fetus and short cervix at 20–24 weeks of gestation were risk factors for PTB before 34 weeks of gestation. Short cervical length, steroid use, multiple pregnancy and male fetus remained as risk factors for 34 weeks of gestation after multivariate analysis. Low family income (<¥2 million/year), part-time work, history of cervical surgery (cone biopsy), uterine myoma, subchorionic hematoma, lean women (body mass index [BMI], <18.5), cervicovaginal infection (grade I PMN or BV-like), cervical inflammation (IL-8, >360 ng/mL), smoking and alcohol intake were not risk factors for PTB before 34 weeks of pregnancy.

Risk factors for PTB before 37 weeks of gestation (Table 3)

In univariate analysis, low educational level, part-time work, uterine myoma, cervical length, steroid use for only collagen disease or bronchial asthma, and multiple pregnancy were risk factors for PTB before 37 weeks of gestation. In multivariate analysis, low educational level, part-time work, multiple pregnancy and short cervical length were risk factors for delivering a preterm baby before 37 weeks of gestation. However, family income level, uterine myoma, history of cervical surgery (cone biopsy), threatened abortion, subchorionic hematoma, fetal sex, smoking, alcohol intake, BMI of less than 18.5, high cervicovaginal level of IL-8 (>360 ng/mL) and vaginal infection in the first trimester (grade I PMN or BV-like) were not risk factors for PTB before 37 weeks of pregnancy.

Cervical length in relation to PTB

Among the 1365 women, the mean (±standard deviation) cervical length at 20–24 weeks was 42.2 ± 8.5 mm (42.5 ± 8.4 mm for nulliparous women and 38.4 ± 9.6 mm for multiparous women). The percentages of PTB before both 34 and 37 weeks of gestation increased with the progression of cervical shortening (Fig. 2). Fifty percent of women with a short cervix of less than 20 mm delivered their babies before 34 weeks of gestation. Seventy-five percent of women with cervical length of less than 20 mm gave birth before 37 weeks of gestation. The odds ratios according to millimeter of cervical length for PTB before 34 and 37 weeks are shown in Figures 3 and 4, respectively. To reveal the relationship among variables, we removed one variable which showed the largest *P*-value, and repeated this procedure until there were no variables of $P \geq 0.10$. The cervical length was the risk factors for PTB before both 34 and 37 weeks of gestation ($P = 0.0000$, odds ratio [OR] = 0.88 [95% confidence interval [CI] = 0.84–0.92]; $P = 0.0001$, OR = 0.95 [95% CI = 0.93–0.98], respectively). We also used a backward stepwise logistic regression analysis for further evaluation. The cervical length at 20–24 weeks of gestation was still the risk factors for PTB before both 34 and 37 weeks of gestation ($P = 0.0000$, OR = 0.89 [95% CI = 0.84–0.94]; $P = 0.0316$, OR = 0.97 [95% CI = 0.94–0.997], respectively).

Discussion

This is the first multicenter, prospective, observational study to detect the risk factors for PTB in Japan. Most participants in this study were women with low-risk pregnancy. Thus, the percentages of preterm delivery in this study before 32 and 37 weeks of pregnancy were 0.3% and 7.5%, respectively, which are not much higher than the levels of those delivered in Japan from 2008–2010 (0.7% and 5.7%, respectively).

The risk of PTB before 34 and 37 weeks in women with cervical length at 20 to 24 weeks of gestation decreased by approximately 11% and 5% for each additional millimeter of cervical length (OR = 0.89 [95% CI = 0.84–0.94] and OR = 0.95 [95% CI = 0.92–0.98], respectively). Werner *et al.*[21] reported that, in low-risk pregnancies, universal transvaginal cervical length ultrasonography appears to be a cost-effective strategy under a wide range of clinical circumstances. Our results support Werner's assertion.[21]

In our study, part-time work was a risk factor for PTB before 37 weeks of gestation. Part-time workers delivered preterm babies more frequently than full-time workers (13.5% vs 7.1%; $P < 0.003$). A study in the USA showed that part-time work (≤20 h/week) was reported to be associated with a lower risk of preterm labor.[6] The low levels of job stress among part-time workers may be attributable to limited exposure to physical job stress. Noborisaka *et al.*[22] showed that the levels of decision latitude in female temporary employees were lower than those in permanent employees. Kobayashi *et al.*[23] also stated that part-time employees experience more job insecurity and poorer prospects for promotion than full-timers.

In Japan, the number of part-time working women has been increasing to help with family expenses. Many pregnant part-timers are not in a situation where they can have an irregular visit without hesitation whenever they had irregular uterine contractions or they had yellowish discharge. Our data may suspect

Table 3 Risk factors for preterm birth before 37 weeks of gestation

			Univariate analysis			Multivariate analysis		
			OR	95% CI	*P*	OR	95% CI	*P*
Educational level	1.	Junior high school	12.86	1.79–92.26	0.00105**	16.03	1.89–136.31	0.0111*
	2.	Others						
Family income	1.	Less than 2 million yen/year	1.05	0.25–4.52	0.94	—	—	—
	2.	Others						
Employment	1.	Part-timer	2.06	1.26–3.35	0.00311**	2.54	1.28–5.07	0.0080**
	2.	Others						
History of cervical surgery (cone biopsy)	1.	Yes	0.74	0.10–5.62	0.77	—	—	—
	2.	No						
Uterine myoma	1.	Yes	2.44	1.20–4.54	0.01085*	2.13	0.78–5.78	0.14
	2.	No						
Threatened abortion	1.	Yes	2.15	0.95–3.41	0.07	—	—	—
	2.	No						
Subchorionic hematoma	1.	Yes	2.15	0.73–6.32	0.15	—	—	—
	2.	No						
Cervical length at 20–24 weeks <25 mm	1.	Yes	9.24	4.03–21.15	0.0000**	0.95†	0.92–0.98†	0.0016**
	2.	No						
Steroid use for collagen disease or bronchial asthma	1.	Yes	6.42	1.16–35.48	0.01443*	8.68	0.79–94.98	0.0767
	2.	No						
Multiple pregnancy	1.	Yes	67.18	29.70–151.95	0.0000**	53.52	18.56–154.35	0.0000**
	2.	No						
Fetal sex	1.	Male	1.23	0.82–1.86	0.32	—	—	—
	2.	Female						
Body mass index <18.5	1.	Yes	0.68	0.42–1.09	0.11	—	—	—
	2.	No						
Smoking during pregnancy	1.	Yes	0.97	0.29–3.20	0.96	—	—	—
	2.	No						
Smoking before pregnancy	1.	Yes	0.9	0.57–1.42	0.66	—	—	—
	2.	No						
Alcohol intake during pregnancy	1.	Yes	0.43	0.06–3.19	0.39	—	—	—
	2.	No						
Alcohol intake before pregnancy	1.	Yes	1.1	0.73–1.65	0.65	—	—	—
	2.	No						
Gram staining at 8–12 weeks: Grade I PMN or BV-like	1.	Yes	0.96	0.64–1.44	0.85	—	—	—
	2.	No						
Chlamydial infection	1.	Yes	N/A	N/A	N/A	—	—	—
	2.	No						
Candida infection	1.	Yes	1.48	0.66–3.33	0.34	—	—	—
	2.	No						
IL-8 level at 20–24 weeks: >360 ng/mL	1.	Yes	0.48	0.25–0.92	0.02418*	0.32	0.15–0.69	0.0036**
	2.	No						

**$P < 0.01$; *$P < 0.05$. †OR (95% CI) according to mm of cervical length. BV, bacterial vaginosis; CI, confidence interval; IL, interleukin; N/A, no one had premature delivery; OR, odds ratio; PMN, polymorphonuclear leukocytes.

that some psychosocial stress in relation to part-time employment facilitate uterine contraction.

The relationship between PTB and uterine leiomyoma is based upon observational studies.[24,25] The presence of larger fibroid (>6 cm) appears to be the factor that best correlates with an increased risk of PTB.[24] Our study showed that uterine myoma was a risk factor for PTB before 37 weeks of gestation in univariate analysis, but not in multivariate analysis. This discrepancy may be due to the small number of cases ($n = 65$).

There is some evidence that women with certain autoimmune diseases, such as autoimmune thyroid disease and inflammatory bowel disease, are at

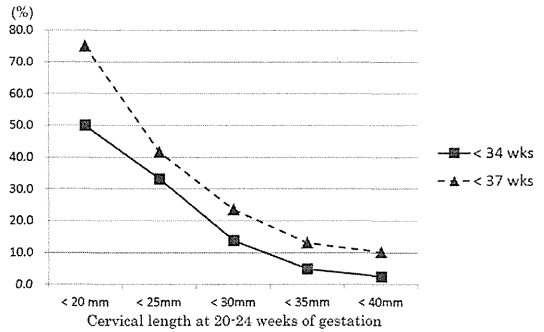

Figure 2 Observed frequency of preterm delivery before 34 weeks (solid line) and 37 weeks (dashed line) according to cervical length measured by transvaginal ultrasonography at 20–24 weeks.

Cervical length	< 20 mm	< 25mm	< 30mm	< 35mm	< 40mm
Preterm delivery (< 34wks)	6	6	10	10	12
Total delivery	12	18	72	206	479
% of preterm delivery (< 34wks)	50.0	33.3	13.9	4.9	2.5

Cervical length	< 20 mm	< 25mm	< 30mm	< 35mm	< 40mm
Preterm delivery (< 37 wks)	9	10	17	27	49
Total delivery	12	24	72	206	479
% of preterm delivery (< 37 wks)	75.0	41.7	23.6	13.1	10.2

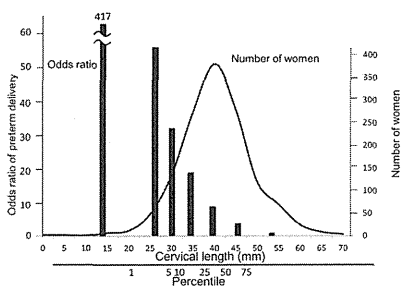

Figure 3 Distribution of subjects among percentiles for cervical length measured by ultrasonography at 20–24 weeks of gestation (solid line) and odds ratio of preterm delivery before 34 weeks of gestation according to percentiles for cervical length (bars).

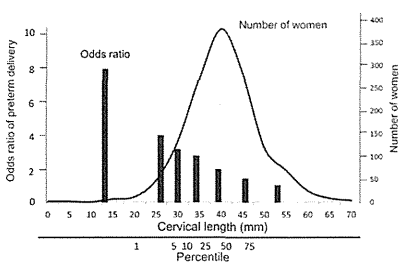

Figure 4 Distribution of subjects among percentiles for cervical length measured by ultrasonography at 20–24 weeks of gestation (solid line) and odds ratio of preterm delivery before 37 weeks of gestation according to percentiles for cervical length (bars).

increased risk for spontaneous PTB.[26,27] In our study, steroid therapy during pregnancy for asthma or collagen disease was a risk factor for PTB before 34 and 37 weeks. The association of premature labor with autoimmune function may be the consequence of abnormalities in normal fetal–placental tolerance, leading to uterine activation and labor.[28]

A number of reports have documented the relationship of a male fetus to PTB.[29–31] Yeganegi *et al.*[32] reported that lipopolysaccharide increased the output of tumor necrosis factor-α, IL-10 and prostaglandin-endoperoxide synthase-2 with a greater response in male placentae compared with female placentae and suggested that there is a greater synthesis of active prostaglandins in the placentae with male fetuses

in a state of inflammation, which may explain the higher incidence of PTB reported for males. Our finding provides further support for the concept that women with a male fetus tend to deliver preterm babies.

Although there is some suggestion that treatment for BV before 20 weeks of gestation may reduce the risk of PTB,[33] this study's data do not suggest that women with BV-like microflora tend to deliver preterm babies. This is possibly because individual centers treated BV-positive women under the diagnosis of BV, irrespective of the result of Gram staining at 8–12 weeks in this study. Alternatively, BV may not be a risk factor for PTB in Japanese pregnant women.

There are some limitations to this study. First, the number of cases enrolled in our study was lower than expected. As a result, we could not extract many variables showing a significant difference. In future, a larger prospective study with many participating obstetricians across the country should be undertaken to identify many risk factors for PTB.

In conclusion, we must pay more attention to women who are of low educational level and part-time workers, and women who have multiple pregnancy, short cervix, steroid use for collagen disease or bronchial asthma, and male fetus to reduce the prevalence of PTB.

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References

1. Simhan HN, Cartis SN. Prevention of preterm delivery. *N Engl J Med* 2007; **357**: 477–487.
2. Goldenberg RL, Culhane JF, Iams JD, Romero R. Epidemiology and causes of preterm birth. *Lancet* 2008; **371**: 75–84.
3. Goldenberg RL, Cliver SP, Mulvihill FX *et al.* Medical, psychosocial, and behavioral risk factors do not explain the increased risk for low birth weight among black women. *Am J Obstet Gynecol* 1996; **175**: 1317–1324.
4. Langhoff-Roos J, Kesmodel U, Jacobsson B, Rasmussen S, Vogel I. Spontaneous preterm delivery in primiparous women at low risk in Denmark: Population based study. *Br Med J* 2006; **332**: 937–939.
5. Biernacka JB, Hanke W, Makowiec-Dabrowska T, Makowska Z, Sobala W. Occupation-related psychosocial factors in pregnancy and risk of preterm delivery. *Med Pr* 2007; **58**: 961–975.
6. Lawson CC, Whelan EA, Hibert EN, Grajewski B, Spiegelman D, Rich-Edwards JW. Occupational factors and the risk of preterm birth in nurses. *Am J Obstet Gynecol* 2009; **200**: 51. e1–8.
7. Domingues MR, Matijasevich A, Barros AJ. Physical activity and preterm birth: A literature review. *Sports Med* 2009; **39**: 961–975.
8. Niedhammer I, O'Mahony D, Daly S, Morrison JJ, Kelleher CC, Lifeways Cross-Genaration Cohort Study Steering Group. Occupational predictors of pregnancy outcomes in Irish working women in the lifeways cohort. *Br J Obstet Gynaecol* 2009; **116**: 943–952.
9. Li X, Sunquist J, Kane K, Jin Q, Sunquist K. Parental occupation and preterm births: A nationwide epidemiological study in Sweden. *Paediatr Perinat Epidemiol* 2010; **24**: 555–563.
10. Ronda E, Moen BE, García AM, Sanchez-Paya J, Baste V. Pregnancy outcomes in female hairdressers. *Int Arch Occup Environ Health* 2010; **83**: 945–951.
11. Zhu P, Tao F, Hao J, Sun Y, Jiang X. Prenatal life events stress: Implications for preterm birth and infant birthweight. *Am J Obstet GYnecol* 2010; **203**: 34. e1–8.
12. Di Renzo GC, Giardina I, Rosati A, Clerici G, Torricelli M, Petraglia F. the Italian Preterm Network Study Group: Maternal risk factors for preterm birth: A country-based population analysis. *Eur J Obstet Gynecol Reprod Biol* 2011; **159**: 342–346.
13. Rours GIJ, Duijts L, Moll HA *et al. Chlamydia trachomatis* infection during pregnancy associated with preterm delivery: A population-based prospective study. *Eur J Epidemiol* 2011; **26**: 493–502.
14. Shiozaki A, Matsuda Y, Hayashi K, Satoh S, Saito S. Comparison of risk factors for major obstetric complications between Western countries and Japan: A case-cohort study. *J Obstet Gynaecol Res* 2011; **37**: 1447–1454.
15. Luo L, Ibaragi T, Maeda M *et al.* Interleukin-8 levles and granulocyte counts in cervical mucus during pregnancy. *Am J Reprod Immunol* 2000; **43**: 78–84.
16. Verstraelen H, Verhelst R, Roelens K *et al.* Modified classification of Gram-stained vaginal smears to predict spontaneous preterm birth: A prospective cohort study. *Am J Obstet Gynecol* 2007; **196**: 528.e1–6.
17. Goldenberg RL, Iams JD, Mercer BM *et al.*, for the National Institute of Child Health and Human Development Maternal-Fetal Medicine Units Network. The preterm prediction study: Sequential cervical length and fetal fibronectin testing for the prediction of spontaneous preterm birth. *Am J Obstet Gynecol* 2000; **182**: 638–643.
18. Guzman ER, Walters C, Ananth CV *et al.* A comparison of sonographic cervical length parameters in predicting spontaneous preterm birth in high-risk singleton gestations. *Ultrasound Obstet Gynecol* 2001; **18**: 204–210.
19. Owen J, Yost N, Berghella V *et al.*, for the National Institute of Child Health and Human Development. Maternal-Fetal Medicine Units Network: Mid-trimester endovaginal Sonography in women at high risk for spontaneous preterm birth. *JAMA* 2001; **286**: 1340–1348.
20. Berghella V, Talucci M, Desai A. Does transvaginal sonographic measurement of cervical length before 14 weeks predict preterm delivery in high-risk pregnancies? *Ultrasound Obstet Gynecol* 2003; **21**: 140–144.
21. Werner EF, Han CS, Pettker CM *et al.* Universal cervical-length screening to prevent preterm birth: A cost-effectiveness analysis. *Ultrasound Obstet Gynecol* 2011; **38**: 32–37.
22. Noborizaka Y, Ishizaki M, Yamada Y. The relationship between job status, gender and work-related stress amongst

middle-aged employees in an computer manufacturing company. *J Occup Health* 1995; **37**: 167–168.
23. Kobayashi Y, Hirose T, Tada Y, Tsutsumi A, Kawakami N. Relationship between two job stress models and coronary risk factors among Japanese part-time female employees of a retail company. *J Occup Health* 2005; **47**: 201–210.
24. Koike T, Minakami H, Kosuge S *et al*. Uterine leiomyoma in pregnancy: Its influence on obstetric performance. *J Obstet Gynaecol Res* 1999; **25**: 309–313.
25. Davis JL, Ray-Mazumder S, Hobel CJ *et al*. Uterine leiomyomas in pregnancy: A prospective study. *Obstet Gynecol* 1990; **75**: 41–44.
26. Thangaratinam S, Tan A, Knox E, Kilby MD, Franklyn J, Coomarasamy A. Association between thyroid autoantibodies and miscarriage and preterm birth: Meta-analysis of evidence. *Br Med J* 2011; **342**: d2616. doi: 10.1136/bmj.d2616. Review.
27. Bortoli A, Saibeni S, Tatarella M *et al*., Study Group for Inflammatory Bowel Diseases GSMII. Pregnancy before and after the diagnosis of inflammatory bowel diseases: Retrospective case–control study. *J Gastroenterol Hepatol* 2007; **22**: 542–549.
28. Gleicher N. Does the immune system induce labor? Lessons from preterm deliveries in women with autoimmune diseases. *Clin Rev Allergy Immunol* 2010; **39**: 194–206.
29. McGregor JA, Leff M, Orleans M, Baron A. Fetal gender differences in preterm birth: Findings in a North American cohort. *Am J Perinatol* 1992; **9**: 43–48.
30. Zeitlin J, Saurel-Cubizolles MJ, De Mouzo J *et al*. Fetal sex and preterm birth: Are males at greater risk? *Human Reprod* 2002; **17**: 2762–2768.
31. Ingemarsson I. Gender aspects of preterm birth. *Br J Obstet Gynaecol* 2003; **110** (Suppl 20): 34–38.
32. Yeganegi M, Watson CS, Martins A *et al*. Effect of Lactobacillus rhamnosus GR-1 supernatant and fetal sex on lipopolysaccharide-induced cytokine and prostaglandin-regulating enzymes in human placental trophoblast cells: Implications for treatment of bacterial vaginosis and prevention of preterm labor. *Am J Obstet Gynecol* 2009; **200**: 532. e1–8.
33. McDonald HM, Brocklehurst P, Gordon A. Antibiotics for treating bacterial vaginosis in pregnancy. *Cochrane Database Syst Rev* 2007; (1): CD000262. Review.

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STUDY PROTOCOL Open Access

The International Network for Evaluating Outcomes of very low birth weight, very preterm neonates (iNeo): a protocol for collaborative comparisons of international health services for quality improvement in neonatal care

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Abstract

Background: The International Network for Evaluating Outcomes in Neonates (iNeo) is a collaboration of population-based national neonatal networks including Australia and New Zealand, Canada, Israel, Japan, Spain, Sweden, Switzerland, and the UK. The aim of iNeo is to provide a platform for comparative evaluation of outcomes of very preterm and very low birth weight neonates at the national, site, and individual level to generate evidence for improvement of outcomes in these infants.

Methods/design: Individual-level data from each iNeo network will be used for comparative analysis of neonatal outcomes between networks. Variations in outcomes will be identified and disseminated to generate hypotheses regarding factors impacting outcome variation. Detailed information on physical and environmental factors, human and resource factors, and processes of care will be collected from network sites, and tested for association with neonatal outcomes. Subsequently, changes in identified practices that may influence the variations in outcomes will be implemented and evaluated using quality improvement methods.

Discussion: The evidence obtained using the iNeo platform will enable clinical teams from member networks to identify, implement, and evaluate practice and service provision changes aimed at improving the care and outcomes of very low birth weight and very preterm infants within their respective countries. The knowledge generated will be available worldwide with a likely global impact.

Keywords: Very preterm infants, Very low birth weight infants, Neonatal intensive care unit, Neonatal networks, Comparative analysis, Neonates, Quality improvement

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Background

The global incidence of preterm birth is on the rise [1]. In Canada the incidence of preterm birth (<37 weeks gestational age) has increased from 6.3% in 1981 to 7.7% in 2009 [2,3]. Although infants born at a very low birth weight (VLBW, <1500 g) and/or very preterm (VPT, <32 weeks gestational age) make up only 14% of all preterm births in Canada [3], they are of significant public health importance due to their high risk of mortality and childhood morbidities. These morbidities include developmental problems, cerebral palsy, cognitive delay, blindness, and deafness [4,5], with an estimated lifetime cost of CAD$676,800 per preterm infant with permanent disability [6]. Therefore, it is important to identify strategies that will reduce the risk of adverse outcomes suffered by VLBW and VPT infants and improve quality of life for these infants.

Various national neonatal networks, such as the Australia-New Zealand Neonatal Network (ANZNN) [7], Canadian Neonatal Network (CNN) [8], Israeli Neonatal Network (INN) [9], Neonatal Research Network of Japan (NRNJ) [10], Swedish Neonatal Quality Register (SNQ) [11], and UK Neonatal Collaborative (UKNC) [12], have been established to collect data from their constituents and identify trends in the outcomes of VLBW infants and benchmark the performance of their respective centers. Although advances in neonatal care between the 1960s and the 1990s resulted in significant reductions in mortality and morbidity for neonates [13-16], recently some networks, including the CNN, have observed a halt in progress or even worsening of outcomes [13,17-19].

Even for those neonatal networks where continued improvements in outcomes have been reported, there remains significant variation within and between networks. For example, several comparative studies have identified differences in mortality rates in neonates from separate networks, regions, or countries [20-27]. In one such study, Draper et al. reported that among 10 European regions, the overall survival rate for VPT infants varied from 74.8% to 93.2% [21]. More recently, in 2012 population data from the UKNC showed a greater than threefold variation between regional networks in the percentage (range 4.7% to 16.6%) of infants born at <30 weeks gestation and admitted to neonatal units who died at ≤28 days of age [27]. Comparison of selected Australian and Scottish neonatal intensive care units (NICUs) detected a lower risk-adjusted mortality rate for VPT/VLBW infants in Australia compared with Scotland [28].

However, studies of a single or small group of sites are subject to selection bias, which can lead to erroneous conclusions when the results are generalized to the larger population. Furthermore, comparisons of mortality alone may be misleading as mortality may be declining at the cost of increasing morbidities. Measurement of mortality as an indicator of care is also a contentious issue as there are marked variations in practice between countries including initiation or withholding of resuscitation at earlier gestational ages [29]. Thus, this protocol for the International Network for Evaluating Outcomes of Neonates (iNeo) was developed to examine neonatal morbidities in conjunction with mortality using population-based data, and assess variations in practice that impact outcomes between and within countries.

Rationale

Over the past 5 years, collaborations have been initiated between the CNN, NRNJ, ANZNN, and SNQ. The first ever population-based retrospective comparison between countries showed that a composite outcome of mortality or any major morbidity (bronchopulmonary dysplasia [BPD], severe neurological injury, ≥stage 3 retinopathy of prematurity [ROP], nosocomial infection [NI], and ≥ stage 2 necrotizing enterocolitis [NEC]) was lower in VLBW infants in Japan compared with Canada. In-depth analyses revealed higher rates of severe neurological injury, NEC, and NI among NICUs in the CNN, whereas rates of BPD and ROP were higher in NRNJ NICUs [30]. Comparisons between the CNN and the ANZNN for VPT infants identified that while there was no difference in mortality, the ANZNN had significantly lower rates of severe neurological injuries, ROP, NEC, and BPD, but higher rates of early onset sepsis and air leaks and longer mean length of stay [31,32]. Our latest comparisons indicated that rates of adverse outcomes at each gestational age were lower in Sweden compared with Canada (unpublished data).

Differences in the outcomes of VLBW and VPT infants between Canada and other countries could be due to any number of factors including differences in population characteristics, severity of illness, processes of care, or delivery of health care. Informal discussion has confirmed wide variations in these factors between networks. For example, compared with Canada, the use of non-invasive respiratory support is higher in Europe, the use of breast milk is higher in Japan and Scandinavia, and the use of echocardiography by neonatologists for hemodynamic monitoring is routine in Japan. Differences in the type of intervention and process of administration may underlie at least some of the variations in outcomes. In addition, there are extreme variations in health services delivery and receipt. For example, the number of outborn, very preterm infants is significantly lower in the ANZNN compared with the CNN [32]; the use of respiratory therapists is practically non-existent in European countries, whereas they play a prominent role in North American institutions; and shift work is more

prevalent among junior doctors in Europe and Australia [33] compared with Canada.

Given the variation in mortality and morbidity between countries, it is important to first characterize factors underlying these differences, and then identify areas and approaches to improve neonatal care specific to each network. Care provision to VLBW and VPT infants is a highly selective health service where specialized units deliver the majority of such care (approximately 80% of VLBW and VPT infants are admitted to tertiary NICUs), and consumes extensive resources, both in terms of the per-diem cost of caring for such a neonate in the NICU and cumulative lifetime costs. To improve outcomes and reduce health care costs globally, we need to embrace the concept of collaborative sharing and learning, assess the variation in practices between countries/networks, identify evidence-based practices associated with improved outcomes, and apply these practices to deliver optimal health care to fragile neonates.

Currently, informal and indirect comparisons can be made from the reports published by each national network. However, criticisms of such indirect or post-hoc comparisons include lack of adjustment for differences in baseline infant and maternal characteristics, differences in definitions of outcomes and their measurement, and variations in physical, environmental, and human factors (e.g. training system and associated working conditions of physicians on duty day and night, differences in nursing care and nurse:beds ratio, differences in regionalization system, and the rate of maternal transfer for extremely preterm fetuses). A system of data standardization and an understanding of the context for comparison are urgently needed to enable valid comparisons between networks. This can only be achieved through an international collaboration where the knowledge users and decision makers are involved from the start of the process and continuously through to knowledge translation. Analyses of network-level data using all eligible infants will provide a more accurate estimate of the effectiveness of an intervention in a pragmatic setting, rather than just a measure of efficacy proven in a controlled study setting.

Network objectives

The specific aims of iNeo are to:

- 1. Compare outcomes for infants born with VLBW (weighing <1500 g) and VPT (<32 weeks gestation) among eight national neonatal networks spanning nine countries.
- 2. Identify site-level physical, human, and environmental characteristics, as well as care practices that are associated with variations in outcomes.
- 3. Identify clinical and organizational practice improvements relevant to each network.
- 4. Implement and continually evaluate the impact of evidence-based clinical and organizational practice changes in NICUs within the iNeo networks.

The establishment of the iNeo collaboration will enable the following: i) collection and integration of individual-level data from population-based networks on outcomes, characteristics, practices, and culture of the member sites; ii) evaluation of the impact of practice and outcome variations to identify the best models of health service delivery (incorporating medical and other extraneous factors); iii) feedback to units of their standing in reference to each and all other networks; iv) empowerment of units to embrace implementation of evidence-based practice changes for quality improvement; and v) performance of ongoing cycles of translating knowledge-to-action through continuous auditing. Ultimately, this will improve outcomes for VLBW infants across the iNeo member networks.

Methods/design

Overview

The comparison of neonatal mortality and morbidity between the eight member networks will be conducted using four years of retrospective data collected between January, 2007 and December, 2010. Subsequently, a strategy will be designed to collect additional data and assess differences in physical, environmental, and human characteristics, and care practices associated with variations in outcomes between networks. Once identified, clinical and organizational practice improvements will be implemented within networks using the Evidence-based Practice for Quality Improvement (EPIQ) method [34,35]. The effect of practice change implementation will be measured using ongoing data collection within each network. The total study period will be five years (January 2013 to December 2017). Comparison between the networks will be completed by early 2014, associations between external factors/care practices and outcomes identified by the end of 2014, and selected practice changes implemented by mid 2015. This will be followed by a two and a half-year period of continuous quality improvement within the networks.

Participating networks

The following neonatal networks have agreed to participate in the iNeo project: Australia-New Zealand Neonatal Network (ANZNN), Canadian Neonatal Network (CNN), Israeli Neonatal Network (INN), Neonatal Research Network of Japan (NRNJ), Spanish Neonatal Network (SEN1500), Swedish Neonatal Quality Register (SNQ), Swiss Neonatal Network (SNN), and UK Neonatal Collaborative (UKNC) (see Table 1). Overall, this project will be collecting data from a total of 251 NICUs in nine countries caring for approximately 23,000 to 24,000 VLBW neonates per year. All the participating networks have a common mandate to collect, analyze, and benchmark performance and outcomes of their respective NICUs. We have carefully avoided networks that only include highly specialized units in order to obtain robust population-based estimates. All participating networks have confirmed the feasibility of data collection from >75% of all VLBW and VPT infants born within their country. The approximately 25% of infants missing from some of the networks are those considered to be at the higher end of maturity (>1300 g birth weight or >30 weeks gestation) who do not require intensive care support. These infants are relatively stable and do not represent a significant burden to NICUs or health care services in general.

Database variables

A detailed review of all the data items collected by each of the participating networks has been conducted and the elements common to all networks (e.g. gestational age, birth weight, sex, etc.) included in a minimum dataset (see Additional file 1 for full list of data variables). Data items that are collected by all networks in slightly different formats (e.g., nosocomial infection, which can be defined by using a cut-off of 2 days, 3 days, or 7 days) have been standardized across all the networks by consensus of the network directors. Some networks already extract data from their databases according to the iNeo definitions, while others have agreed to redefine their original data formats as an ongoing process to ensure consistency and facilitate comparisons over time. The variable definitions have been mapped to the ICD-10 [36] and SNOMED [37] dictionaries.

Ethics, data collection, and dissemination

All participating networks have obtained ethics/regulatory approval or the equivalent from their local granting agencies to allow for de-identified data to be sent to the iNeo Coordinating Centre at the Maternal-Infant Care Research Centre, Mount Sinai Hospital, Toronto, Canada. The Coordinating Centre has been granted Research Ethics Board approval for the development, compilation, and hosting of the iNeo dataset, and all networks have signed data transfer agreements with the iNeo Coordinating Centre. Privacy and confidentiality of patient and unit-related data will be of prime importance to the iNeo collaboration, and data collection, handling, and transfer will be performed in accordance with the Canadian Privacy Commissioner's guidelines, the Personal Information Protection and Electronic Documents Act, and any other local rules and regulations. No data identifiable at the patient level will be collected or transmitted, and only aggregate data will be reported. For all stages of the project, participating units will be assigned a code by their own network prior to data transfer into the iNeo dataset so that units remain anonymous within the iNeo collaborative. Following data analysis, findings will be disseminated within networks by their own network coordination team and not by the iNeo central team.

Following completion of the study in 2017, the data will be kept at the iNeo Coordinating Centre for a further two years before being returned to the originating networks unless otherwise agreed by the member networks.

Table 1 Characteristics of networks participating in the International Network for Evaluating Outcomes of Neonates (iNeo)

Network	Australia and New Zealand Neonatal Network	Canadian Neonatal Network	Israeli Neonatal Network	Neonatal Research Network Japan	Spanish Neonatal Network	Swedish Neonatal Quality Register	Swiss Neonatal Network & Follow-Up Group	UK Neonatal Collaborative
Country	Australia and New Zealand	Canada	Israel	Japan	Spain	Sweden	Switzerland	UK (England)
Level III NICUs in the country	23 + 6	30	23	93	n/a	7	9	45
Level III NICUs in the network	29	30	23	73	36	7	9	44
Number of inhabitants	Australia: 23 million NZ: 4.4 million	34 million	7.9 million	126 million	47 million	9.5 million	8 million	52 million
Number of births/year	Australia: 300,000 NZ: 60,000	380,863	166,000	1,071,304	497,023	110,000	80,000	687,000
Number of eligible NICU admissions/year (<32 wks gestation/<1500 g)	3,500	2,700	1,500	3,700	2,600	900	800	7,700

Comparisons of neonatal outcomes between networks

Outcomes

The primary outcome for comparison between the networks will be a composite indicator of mortality or any of the four major neonatal morbidities (severe neurological injury, severe ROP, NEC, and BPD). Mortality will be defined as death due to any cause prior to discharge home. Severe neurological injury will be defined as ≥ stage 3 intraventricular hemorrhage (IVH) with ventricular dilatation according to the criteria of Papile et al. [38], or parenchymal injury (including periventricular leukomalacia) with or without IVH. Severe ROP will be defined as ≥ stage 3 according to the International Classification [39], or need for laser surgery or intraocular injections of anti-vascular endothelial growth factor agents. NEC will be defined as ≥ stage 2 according to Bell's criteria [40] and BPD as oxygen requirement at 36 weeks post-menstrual age [41].

Secondary outcomes to be compared among iNeo member networks will include the individual morbidities of the composite outcome, as well as nosocomial infection defined as culture-proven sepsis (blood or cerebrospinal fluid positive for pathogenic organism) at >3 days or 72 hours postnatal age [42], patent ductus arteriosus requiring pharmacological treatment and/or surgical ligation, receipt of delivery room cardiopulmonary resuscitation, air leak syndrome, and resource utilization (length of stay and length of respiratory support). To account for potential differences in practices regarding discharge home and transfer to Level 2 community units, additional analyses will compare mortality by Day 28 after birth. All outcomes will be expressed as ratios with the denominator equal to all admissions to participating NICUs.

Adjustment for variations in baseline population characteristics between networks

Demographic characteristics and severity of illness are well known to impact neonatal outcomes [43] and are also likely to vary between networks. To prevent bias, these potential confounders will be controlled in analyses comparing network-level outcome rates. The common minimum dataset includes important predictors, such as gestational age, sex, plurality of pregnancy, and receipt of antenatal corticosteroids, which will be used to adjust analyses as appropriate. In addition, most networks collect various measures of 'severity of illness', such as CRIB [44], SNAPPE-II [45], or TRIPS [46] scores. These will be standardized within each network (assigned a score between 0 and 1) and adjusted for in analyses.

Descriptive analyses of baseline factors

The distribution of infant characteristics and network-level broad organizational structural features will be summarized as counts and percentages for categorical variables and using the mean and standard deviation, or the median and interquartile range for continuous variables. The data will be compared among all networks using the Chi-square test for categorical and ANOVA F-test or Mood's median test for continuous variables.

Comparisons between networks

For the primary composite outcome, each of its components and the additional secondary outcomes, initial crude rates, and associated 95% and 99% confidence intervals will be calculated and graphically displayed using 'caterpillar plots' to visually identify differences between networks. To adjust for multiple baseline characteristics, standardized outcome ratios will be computed using the 'indirect standardization' approach. Each network's observed rate will be compared with the expected rate based on the total sample from all other networks to identify networks with rates significantly above or below average. For each outcome, the expected number of events will be computed as the sum of predicted probabilities from a multivariable model (logistic regression or zero inflated negative binomial models based on data distribution) derived using data from all other networks with adjustment for confounders. Network standardized outcome ratios will be graphically displayed using 'funnel' plots with 95% and 99% prediction intervals for comparison between networks.

A global comparison, as well as pair-wise comparisons between networks, will be performed using multivariate regression models adjusted for confounders. Statistical models will employ generalized estimating equations to adjust analyses for clustering of infants within networks. In addition, hierarchical random-effects regression models will be used to allow for variation at the network and unit level. Statistical significance will be evaluated by applying a Bonferroni correction to account for multiple pair-wise comparisons.

Statistical power for outcome comparisons

With retrospective data from 251 NICUs collected over four years (2007–2010), analyses (two-sided tests) comparing Canada (10,800 admissions) with all other networks (82,800 admissions), for example, will be able to detect rate differences of 0.004 to 0.02 for a range of outcome rates (1% to 40%) with statistical power of 80% assuming 5% type I error rate. Similar analyses comparing Canada with one other network (3,200 to 30,800 admissions) will be able to detect rate differences of 0.007 to 0.03.

Association of site characteristics and practices with outcomes

To identify factors contributing to outcome variation between networks, detailed information will be obtained on health service provision, including units' physical layout, environmental characteristics, human factors, and management practices at the national and site level. The type of data and strategy for collecting this information will be determined following the comparison of outcomes between networks to target identified problem areas and evaluate the culture, context, and practices of each network. Factors with possible impact on outcome differences between and within networks will be ascertained using a variety of tools, such as surveys, recurring questionnaires, and in specific instances, site visits to explore details if permitted.

The data will be pooled across sites and networks, and statistical analyses will identify factors significantly associated with outcomes. Through a collaborative process, findings will be discussed with members of participating networks to select physical and environmental factors, human and resource factors, or processes of care that can be modified through a quality improvement process. Each network will then implement practice changes within these three main target areas according to their outcome priorities and the constraints of their respective health care systems.

Physical and environmental factors

For preterm infants, adaptation to the environment is crucial for their survival, wellbeing, and development. The physical environment of the NICU is significantly different from the in-utero environment and contains a wide range of sensory stimuli that a preterm infant would not be exposed to if carried to term [47]. There has been wide debate as to the optimal physical characteristics of a NICU in relation to outcomes for VLBW infants. Several units that have implemented a single infant per room design in place of the more traditional open multi-patient rooms have reported improvements in outcomes, but impact on staff satisfaction and work-efficiency remains unclear [48,49]. Higher physical demands and workloads placed on nurses could negatively affect the level of care provided. Additional key physical characteristics include internal and external noise [50,51], temperature control, exposure to light [52,53], practice of developmentally supportive care [54], provision and extent of family-centered care, provision and extent of breastfeeding support, potential for continuous parental involvement, as well as training and preparation for discharge home.

Physical characteristics will be assessed by conducting a snapshot survey of units within the iNeo networks. The survey will be developed, piloted, and implemented in collaboration with the iNeo Scientific Advisory Committee by iNeo researchers with experience investigating the extraneous factors that may impact quality of care.

Human and resource factors

Human factors and available resources represent another aspect of care provision possibly associated with differences in outcomes. However, associations between human and resource factors and neonatal outcomes have not been thoroughly investigated, particularly not on a national scale. Human factors include staffing in relation to day and night shifts [33,55], weekdays versus weekends [56], ratio of nurses to patients [57], pattern of work for medical and nursing staff (hours on call, total duration of active duty time over 4 week period, etc.), number and types of trainee doctors, allied healthcare personnel coverage, constitution of attending team for high-risk births, and relative expertise of the health care providers attending resuscitation of extremely preterm infants considering their overall experience in direct patient care, training, and research.

Neonatal outcomes are also impacted by resource availability and utilization, specifically volume and capacity. Units with high volume are reported to have better outcomes compared with units with low volume, possibly due to relatively increased staff experience [58,59]; however, it has also been noted that low volume units may be less crowded and have reduced rates of complications [60]. Alternatively, these differences may be secondary to centralization of care rather than volume, as seen in data from Finland [61]. Similarly, units functioning at >90% capacity at all times, irrespective of volume, may have different outcomes compared with units operating at lower capacity.

Data on human factors and resource utilization will be collected using snapshot surveys administered at the unit level. Due to likely variations from year to year, data on human factors and resource utilization will be collected on an annual basis using electronic tools (such as recurring auto-filled surveys based on previous responses so as to only report changes), and while the data may not capture variation in the daily activity levels or acuity in the unit, this will represent the average condition.

Care-provision factors

Clinical practices represent the third and possibly most important set of characteristics that likely contribute to variation in outcomes. Variations in clinical practices are well known among neonatal communities [8]; however, no systematic prospective approach has determined, compared, and benchmarked variations associated with outcomes. Some of the key practice variations between centers and networks include referral practices (inborn vs. outborn) [62-64], differential use of the type of initial respiratory support [65-67], types and timings of surfactant administration [68], fluid management [69], timing of initiation of parenteral nutrition [70], use of donor milk, management of patent ductus arteriosus [71], availability

and use of echocardiography, use of prophylactic interventions [72] (e.g., probiotics, high frequency oscillatory ventilation, phototherapy, and L-arginine), and the scope of involvement of parents.

Specific to each secondary outcome we will identify 'top' performing networks and networks with significant room for improvement. Subsequently, working groups of interested stakeholders from each network will be formed to determine methods to identify possible care provision practices related to such variations. Study methods will be similar to those described earlier, and will include annual snapshot surveys of each unit, detailed questionnaires specific to practices (e.g. parental presence, use of donor milk, diagnosis and management of hypotension, etc.), and in certain instances of outstanding success, a site visit with structured exploration of the practices in question. All methods of exploration will be conducted with directions from the iNeo Governing Board and Scientific Advisory Committee to protect privacy and confidentiality. Because individual unit information will not be disclosed to the iNeo Coordinating Centre, individual networks will be asked to identify willing members for such participation.

Statistical analyses and power for identification of practice and service variation

Associations of clinical management practices and other external factors with outcomes will be assessed under the general framework of individual patient-level data meta-analyses. Random-effects models with adjustment for confounding variables and important risk factors will provide estimates of association and quantify residual variation due to unknown or unmeasured unit-specific and network-level factors. These analyses will identify treatment practices and health care services with significant impact on outcomes, which subsequently can be targeted for implementation or improvement by specific units or networks. This information along with details of the practices/factors will be made available to initiate discussion within the iNeo community regarding data-informed, evidence-linked potentially better practices. Analyses (two-sided tests) based on 10,000 yearly admissions evaluating impact of treatment/practices (assuming 50% exposure) on outcomes (incidence 1% to 40%) will be able to detect relative risks of 1.6 to 1.1 with statistical power of 80% and 5% type I error rate. This is a conservative power calculation based on data expected to be collected in a one-year timeframe.

Implementation and evaluation of practice changes to improve outcomes

Practices identified as being associated with an improvement in outcomes will be proposed to network sites for implementation using the continuous cycle of application and evaluation central to the EPIQ method [34,35]. Quality improvement using EPIQ methodology has been implemented in Canadian NICUs for the last 10 years. It is based on three pillars: (1) the use of all available evidence on a particular intervention from the published scientific literature, (2) analysis of each institute's baseline data to identify hospital-specific practices for targeted intervention, and (3) the use of a network to share the results of quality improvement for the purpose of collaborative learning. The EPIQ method utilizes local context and allows customization of interventions and implementation strategies to maximize improvement potential at each institute. This is conducted in conjunction with leadership and peer support from network members [34,35].

Our plan for the iNeo network is to expand the EPIQ approach to an international level. We will advocate incorporation of several cycles of practice change implementation, evaluation, monitoring, and collaborative learning within each unit over the course of two and a half years. The online ViviWeb Virtual Research Community (https://meta.cche.net/viviweb/default.asp) will be used to facilitate collaboration between networks. Based on our experiences and preliminary results implementing practice changes in Canada, and following discussion with the NRNJ, we anticipate that regular and productive dialogue will significantly benefit many of the participating NICUs.

The practice changes implemented by individual units within networks will be evaluated every 6 to 12 months depending upon each centre's capabilities to collect and submit data. In addition to outcome indicators, process indicators will be developed based on the specific interventions implemented. These indicators will measure the short-term impact of practice change. For example, an intervention targeting early surfactant administration to reduce BPD will have process indicators for the time of first surfactant administration and the proportion of babies who received surfactant within the first 30 minutes after birth. The outcome of interest for this intervention will be reduction in the incidence of BPD. Safety and outcome improvements will be monitored within each unit and network using control charts and Chi-square tests for differences in outcome rates from baseline. Multivariable logistic regression analyses will pool data from units within each network to assess changes in outcomes over time with adjustment for potential confounders and important risk factors, and accounting for clustering.

Long-term neurodevelopmental follow-up

The members of iNeo have agreed that while the present initiative should focus on ascertaining outcomes prior to discharge from the NICU, the longer-term goal should be to assess and improve neurodevelopmental outcomes of VLBW and VPT infants at two to three years of age.

Presently, five networks (CNN, NRNJ, NDAU, SNN, and ANZNN) follow and collect data from their infants up to two to three years of age with one more network in the planning stages of follow-up data collection (SNQ). The remaining networks have expressed interest in long-term follow-up, and will explore the possibility of collecting these data. For available follow-up data, extraneous factors, and process of care factors during NICU stay will be examined in relation to outcomes at two to three years of age. A composite severe adverse outcome will be defined as mortality or severe morbidity, including non-ambulatory cerebral palsy, developmental indices more than two standard deviations below the mean, legal blindness, or deafness requiring amplification. This will require development of a follow-up dataset (similar to the NICU minimum dataset) for the long-term neurodevelopmental outcomes.

Secondary research questions

In order to foster a true international collaboration, the data collected and housed at the iNeo Coordinating Centre will be available to all iNeo member networks and iNeo-affiliated investigators after the principal analyses are completed. The iNeo database will be available to iNeo-affiliated investigators, including trainees, wishing to examine new research questions/hypotheses. Requests for data will need to be sent to the iNeo Coordinating Centre for discussion and approval by the iNeo Scientific Advisory Committee. In the initial stages of the iNeo collaboration, analysis of the dataset in question will be performed at the iNeo Coordinating Centre and the results sent to the requesting investigator. In the later stages, limited datasets may be released to an investigator using a secure electronic portal system. In all publications, the final author will be 'the International Network for Evaluating Outcomes of Neonates (iNeo)'. For the analyses detailed in this protocol, the author list will include representatives of all eight networks. For additional projects, authors will be those individuals who meet the criteria for authorship as laid out by the ICJME. All publications will include a list of the member networks in the acknowledgements.

Discussion

The iNeo collaboration will be the first multi-national network to examine population-based data. Findings from this international collaboration generated using extensive data will provide strong and novel evidence regarding practices contributing to outcome variation with broad relevance to NICUs within iNeo and worldwide. This is particularly true for the investigation of the environmental, human, and physical factors that impact neonatal outcomes. The majority of current literature relates to single center or regional experiences, whereas data from multiple national networks will provide robust estimates that will allow development of unified recommendations regarding optimal design and staffing of neonatal units.

The nature of the information that will be generated and the resources available within the collaborative will put iNeo in a unique position to implement global change to improve neonatal outcomes. Neonatal outcomes and NICU care practices will likely vary significantly between networks and there are many factors that may underlie these variations. The initial findings from the comparative analysis may not be welcomed by all units, and recommendations for practice changes that require extensive change or high financial input, such as additional staff to attend births or changes to unit layout, may be met with resistance. In answer to this, the most persuasive element of the iNeo collaboration will be the strength of the evidence produced from the data, the pragmatic nature of the results, and higher degree of statistical precision due to the large sample size.

In addition to the strength of the data, a high level of collaboration between network members will provide a mechanism to address barriers to change and ensure the knowledge gained is effectively implemented to improve neonatal outcomes. Working together we will ensure that all factors that contribute to a target outcome are identified and evaluated. Once identified, the process for exploration of extraneous factors will be supervised by the iNeo Director and Scientific Advisory Committee to ensure that all suggested practice changes can be tailored to networks depending on the presence or absence of certain baseline covariates. Although the individual network directors will be primarily responsible for driving change within their networks, iNeo will also provide various activities and mechanisms to facilitate practice change. This will include access to in-person and online training, site visits between networks, effective dissemination of information, and liaison with policy makers in member countries.

The iNeo collaboration will also act as a platform whereby other NICUs and established networks or networks in the preliminary phase of development can access evidence regarding impact of practices on outcomes, and approaches for collaborative learning and practice improvement in neonatology. As such, initial discussions with neonatal units in India, China, South America, and Taiwan have been productive and these networks are planning to assess and apply the results of the iNeo collaboration.

In summary, the iNeo collaboration will serve as a strong international platform for neonatal-perinatal health services research in VLBW and VPT infants. The evidence obtained using the iNeo platform will enable clinical teams from member networks to identify,

implement, and evaluate practice and service provision changes aimed at improving the care and outcomes of VLBW and VPT infants within their respective countries. The knowledge generated, assembly of expertise, and pool of resources will be available worldwide with a likely global impact.

Additional file

Additional file 1: iNeo data variables for collection with explanatory notes. Description: List of the data variables that will be collected and analyzed during the project described in the iNeo protocol.

Abbreviations

ANZNN: Australia-New Zealand Neonatal Network; BPD: Bronchopulmonary dysplasia; CNN: Canadian Neonatal Network; EPIQ: Evidence-based Practice for Quality Improvement; iNeo: International Network for Evaluating Outcomes of Neonates; INN: Israel Neonatal Network; IVH: Intraventricular hemorrhage; NEC: Necrotizing enterocolitis; NI: Nosocomial infection; NICU: Neonatal intensive care unit; NRNJ: Neonatal Research Network of Japan; SNN: Swiss Neonatal Network; SNQ: Swedish Neonatal Quality Register: Neonatology; ROP: Retinopathy of prematurity; SEN1500: Spanish Neonatal Network; UKNC: UK Neonatal Collaborative; VLBW: Very low birth weight; VPT: Very preterm.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

PSS conceived of the concept of iNeo, led the protocol design process, and drafted the manuscript. LM designed the statistical analysis plan and participated in the protocol design process. All the remaining authors (SKL, KL, GS, RM, BR, SH, LSF, NM, MA, BD, MF, SK, RH) participated in network and protocol design including reaching consensus on the minimum dataset, and will direct the collection of data, dissemination of knowledge, and implementation of practice changes within their respective networks. All authors read, revised, and approved the final manuscript.

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References

1. Blencowe H, Cousens S, Oestergaard MZ, Chou D, Moller AB, Narwal R, Adler A, Vera GC, Rohde S, Say L, Lawn JE: **National, regional, and worldwide estimates of preterm birth rates in the year 2010 with time trends since 1990 for selected countries: a systematic analysis and implications.** *Lancet* 2012, **379:**2162–2172.
2. Joseph KS, Kramer MS, Marcoux S, Ohlsson A, Wen SW, Allen A, Platt R: **Determinants of preterm birth rates in Canada from 1981 through 1983 and from 1992 through 1994.** *N Engl J Med* 1998, **339:**1434–1439.
3. Statistics Canada: *Births*; 2012. 84F0210X.
4. Kliegman RM, Rottman CJ, Behrman RE: **Strategies for the prevention of low birth weight.** *Am J Obstet Gynecol* 1990, **162:**1073–1083.
5. McCormick MC: **The contribution of low birth weight to infant mortality and childhood morbidity.** *N Engl J Med* 1985, **312:**82–90.
6. Moutquin J, Milot-Roy VI: **Preterm birth prevention: effectiveness of current strategies.** *J Soc Obstet Gynecol Can* 1996, **18:**571–588.
7. Cust AE, Darlow BA, Donoghue DA: **Outcomes for high risk New Zealand newborn infants in 1998–1999: a population based, national study.** *Arch Dis Child Fetal Neonatal Ed* 2003, **88:**F15–F22.
8. Lee SK, McMillan DD, Ohlsson A, Pendray M, Synnes A, Whyte R, Chien LY, Sale J: **Variations in practice and outcomes in the Canadian NICU network: 1996–1997.** *Pediatrics* 2000, **106:**1070–1079.
9. Bader D, Kugelman A, Boyko V, Levitzki O, Lerner-Geva L, Riskin A, Reichman B: **Risk factors and estimation tool for death among extremely premature infants: a national study.** *Pediatrics* 2010, **125:**696–703.
10. Kusuda S, Fujimura M, Sakuma I, Aotani H, Kabe K, Itani Y, Ichiba H, Matsunami K, Nishida H: **Morbidity and mortality of infants with very low birth weight in Japan: center variation.** *Pediatrics* 2006, **118:**e1130–e1138.
11. Fellman V, Hellstrom-Westas L, Norman M, Westgren M, Kallen K, Lagercrantz H, Marsal K, Serenius F, Wennergren M: **One-year survival of extremely preterm infants after active perinatal care in Sweden.** *JAMA* 2009, **301:**2225–2233.
12. Gale C, Santhakumaran S, Nagarajan S, Statnikov Y, Modi N: **Impact of managed clinical networks on NHS specialist neonatal services in England: population based study.** *BMJ* 2012, **344:**e2105.
13. Fanaroff AA, Hack M, Walsh MC: **The NICHD neonatal research network: changes in practice and outcomes during the first 15 years.** *Semin Perinatol* 2003, **27:**281–287.
14. Horwood SP, Boyle MH, Torrance GW, Sinclair JC: **Mortality and morbidity of 500- to 1,499-gram birth weight infants live-born to residents of a defined geographic region before and after neonatal intensive care.** *Pediatrics* 1982, **69:**613–620.
15. Lemons JA, Bauer CR, Oh W, Korones SB, Papile LA, Stoll BJ, Verter J, Temprosa M, Wright LL, Ehrenkranz RA, Fanaroff AA, Stark A, Carlo W, Tyson JE, Donovan EF, Shankaran S, Stevenson DK: **Very low birth weight outcomes of the National Institute of Child health and human development neonatal research network, January 1995 through December 1996. NICHD Neonatal Research Network.** *Pediatrics* 2001, **107:**E1.
16. Hack M, Wright LL, Shankaran S, Tyson JE, Horbar JD, Bauer CR, Younes N: **Very-low-birth-weight outcomes of the National Institute of Child Health and Human Development Neonatal Network, November 1989 to October 1990.** *Am J Obstet Gynecol* 1995, **172:**457–464.
17. Horbar JD, Badger GJ, Carpenter JH, Fanaroff AA, Kilpatrick S, LaCorte M, Phibbs R, Soll RF: **Trends in mortality and morbidity for very low birth weight infants, 1991–1999.** *Pediatrics* 2002, **110:**143–151.
18. Meadow W, Lee G, Lin K, Lantos J: **Changes in mortality for extremely low birth weight infants in the 1990s: implications for treatment decisions and resource use.** *Pediatrics* 2004, **113:**1223–1229.
19. Shah PS, Sankaran K, Aziz K, Allen AC, Seshia M, Ohlsson A, Lee SK: **Outcomes of preterm infants <29 weeks gestation over 10-year period in Canada: a cause for concern?** *J Perinatol* 2012, **32:**132–138.
20. Draper ES, Zeitlin J, Field DJ, Manktelow BN, Truffert P: **Mortality patterns among very preterm babies: a comparative analysis of two European regions in France and England.** *Arch Dis Child Fetal Neonatal Ed* 2007, **92:**F356–F360.
21. Draper ES, Zeitlin J, Fenton AC, Weber T, Gerrits J, Martens G, Misselwitz B, Breart G: **Investigating the variations in survival rates for very preterm infants in 10 European regions: the MOSAIC birth cohort.** *Arch Dis Child Fetal Neonatal Ed* 2009, **94:**F158–F163.
22. Evans N, Hutchinson J, Simpson JM, Donoghue D, Darlow B, Henderson-Smart D: **Prenatal predictors of mortality in very preterm infants cared for in the Australian and New Zealand Neonatal Network.** *Arch Dis Child Fetal Neonatal Ed* 2007, **92:**F34–F40.
23. Field D, Bajuk B, Manktelow BN, Vincent T, Dorling J, Tarnow-Mordi W, Draper ES, Smart DH: **Geographically based investigation of the influence of very-preterm births on routine mortality statistics from the UK and Australia.** *Arch Dis Child Fetal Neonatal Ed* 2008, **93:**F212–F216.
24. Parry GJ, Gould CR, McCabe CJ, Tarnow-Mordi WO: **Annual league tables of mortality in neonatal intensive care units: longitudinal study. International Neonatal Network and the Scottish Neonatal Consultants and Nurses Collaborative Study Group.** *BMJ* 1998, **316:**1931–1935.
25. Richardus JH, Graafmans WC, Verloove-Vanhorick SP, Mackenbach JP: **The perinatal mortality rate as an indicator of quality of care in international comparisons.** *Med Care* 1998, **36:**54–66.
26. Ishii N, Kono Y, Yonemoto N, Kusuda S, Fujimura M: **Outcomes of infants born at 22 and 23 weeks' gestation.** *Pediatrics* 2013, **132:**62–71.
27. UK Chief Medical Officer: *Atlas of Variation in Healthcare for Children and Young People, Annex of the Annual Report of the Chief Medical Officer 2012.* Our Children Deserve Better: Prevention Pays; 2013.
28. Risk adjusted and population based studies of the outcome for high risk infants in Scotland and Australia: International Neonatal Network, Scottish Neonatal Consultants, Nurses Collaborative Study Group. *Arch Dis Child Fetal Neonatal Ed* 2000, **82:**F118–F123.
29. Sexson WR, Cruze DK, Escobedo MB, Brann AW: **Report of an international conference on the medical and ethical management of the neonate at the edge of viability: a review of approaches from five countries.** *HEC Forum* 2011, **23:**31–42.
30. Isayama T, Lee SK, Mori R, Kusuda S, Fujimura M, Ye XY, Shah PS: **Comparison of Mortality and Morbidity of Very Low Birth Weight Infants Between Canada and Japan.** *Pediatrics* 2012, **130:**e957–e965.
31. Hossain S, Shah PS, Lee SK, Darlow B, Sullivan E, Lui K: **Comparison of characteristics and outcomes of very preterm infants admitted to Australia-New Zealand and Canadian neonatal networks [abstract].** *Pediatr Acad Soc* 2012.
32. Shah PS, Hossain S, Lui K, Darlow B, Gallimore V, Lee SK: **Outcomes of outborn very preterm infants in Australia-New Zealand and in Canada [abstract].** *Pediatr Acad Soc* 2012.
33. Abdel-Latif ME, Bajuk B, Oei J, Lui K: **Mortality and morbidities among very premature infants admitted after hours in an Australian neonatal intensive care unit network.** *Pediatrics* 2006, **117:**1632–1639.
34. Lee SK, Aziz K, Singhal N, Cronin CM, James A, Lee DS, Matthew D, Ohlsson A, Sankaran K, Seshia M, Synnes A, Walker R, Whyte R, Langley J, Macnab YC, Stevens B, von Dadelszen P: **Improving the quality of care for infants: a cluster randomized controlled trial.** *CMAJ* 2009, **181:**469–476.
35. Cronin CM, Baker GR, Lee SK, Ohlsson A, McMillan DD, Seshia MM: **Reflections on knowledge translation in Canadian NICUs using the EPIQ method.** *Healthc Q* 2011, **3:**8–16. 14 Spec No.
36. World Health Organization: *International Statistical Classification of Diseases and Related Health Problems 10th Revision (ICD-10)*; 2010. http://apps.who.int/classifications/icd10/browse/2010/en.
37. *International Health Terminology Standards Development Organisation. SNOMED CT*; 2012. *http://www.ihtsdo.org/snomed-ct/*.
38. Papile LA, Burstein J, Burstein R, Koffler H: **Incidence and evolution of subependymal and intraventricular hemorrhage: a study of infants with birth weights less than 1,500 gm.** *J Pediatr* 1978, **92:**529–534.
39. International Committee for the Classification of Retinopathy of Prematurity: **The International Classification of Retinopathy of Prematurity revisited.** *Arch Ophthalmol* 2005, **123:**991–999.
40. Bell MJ, Ternberg JL, Feigin RD, Keating JP, Marshall R, Barton L, Brotherton T: **Neonatal necrotizing enterocolitis. Therapeutic decisions based upon clinical staging.** *Ann Surg* 1978, **187:**1–7.
41. Shennan AT, Dunn MS, Ohlsson A, Lennox K, Hoskins EM: **Abnormal pulmonary outcomes in premature infants: prediction from oxygen requirement in the neonatal period.** *Pediatrics* 1988, **82:**527–532.
42. Freeman J, Epstein MF, Smith NE, Platt R, Sidebottom DG, Goldmann DA: **Extra hospital stay and antibiotic usage with nosocomial coagulase-negative staphylococcal bacteremia in two neonatal intensive care unit populations.** *Am J Dis Child* 1990, **144:**324–329.
43. Shah PS, Ye XY, Synnes A, Rouvinez-Bouali N, Yee W, Lee SK: **Prediction of survival without morbidity for infants born at under 33 weeks gestational age: a user-friendly graphical tool.** *Arch Dis Child Fetal Neonatal Ed* 2012, **97:**F110–F115.
44. Parry G, Tucker J, Tarnow-Mordi W: **CRIB II: an update of the clinical risk index for babies score.** *Lancet* 2003, **361:**1789–1791.
45. Richardson DK, Corcoran JD, Escobar GJ, Lee SK: **SNAP-II and SNAPPE-II: Simplified newborn illness severity and mortality risk scores.** *J Pediatr* 2001, **138:**92–100.
46. Lee SK, Zupancic JA, Pendray M, Thiessen P, Schmidt B, Whyte R, Shorten D, Stewart S: **Transport risk index of physiologic stability: a practical system for assessing infant transport care.** *J Pediatr* 2001, **139:**220–226.
47. Kuhn P, Zores C, Astruc D, Dufour A, Casper C: **[Sensory system development and the physical environment of infants born very preterm].** *Arch Pediatr* 2011, **18**(Suppl 2):S92–S102.
48. Domanico R, Davis DK, Coleman F, Davis BO: **Documenting the NICU design dilemma: comparative patient progress in open-ward and single family room units.** *J Perinatol* 2011, **31:**281–288.
49. Domanico R, Davis DK, Coleman F, Davis BO Jr: **Documenting the NICU design dilemma: parent and staff perceptions of open ward versus single family room units.** *J Perinatol* 2010, **30:**343–351.
50. Darcy AE, Hancock LE, Ware EJ: **A descriptive study of noise in the neonatal intensive care unit. Ambient levels and perceptions of contributing factors.** *Adv Neonatal Care* 2008, **8:**165–175.
51. Liu WF: **Comparing sound measurements in the single-family room with open-unit design neonatal intensive care unit: the impact of equipment noise.** *J Perinatol* 2012, **32:**368–373.
52. Lasky RE, Williams AL: **Noise and light exposures for extremely low birth weight newborns during their stay in the neonatal intensive care unit.** *Pediatrics* 2009, **123:**540–546.
53. Morag I, Ohlsson A: **Cycled light in the intensive care unit for preterm and low birth weight infants.** *Cochrane Database Syst Rev* 2011. CD006982.
54. Als H: **NIDCAP: testing the effectiveness of a relationship-based comprehensive intervention.** *Pediatrics* 2009, **124:**1208–1210.
55. Lee SK, Lee DS, Andrews WL, Baboolal R, Pendray M, Stewart S: **Higher mortality rates among inborn infants admitted to neonatal intensive care units at night.** *J Pediatr* 2003, **143:**592–597.
56. Salihu HM, Ibrahimou B, August EM, Dagne G: **Risk of infant mortality with weekend versus weekday births: A population-based study.** *J Obstet Gynaecol Res* 2012.
57. Mefford LC, Alligood MR: **Evaluating nurse staffing patterns and neonatal intensive care unit outcomes using Levine's Conservation Model of Nursing.** *J Nurs Manag* 2011, **19:**998–1011.
58. Grandi C, Gonzalez A, Meritano J: **[Patient volume, medical and nursing staffing and its relationship with risk-adjusted outcomes of VLBW infants in 15 Neocosur neonatal network NICUs].** *Arch Argent Pediatr* 2010, **108:**499–510.
59. Phibbs CS, Bronstein JM, Buxton E, Phibbs RH: **The effects of patient volume and level of care at the hospital of birth on neonatal mortality.** *JAMA* 1996, **276:**1054–1059.
60. Tucker J: **Patient volume, staffing, and workload in relation to risk-adjusted outcomes in a random stratified sample of UK neonatal intensive care units: a prospective evaluation.** *Lancet* 2002, **359:**99–107.

61. Rautava L, Lehtonen L, Peltola M, Korvenranta E, Korvenranta H, Linna M, Hallman M, Andersson S, Gissler M, Leipala J, Tammela O, Hakkinen U: **PERFECT Preterm Infant Study Group: The effect of birth in secondary- or tertiary-level hospitals in Finland on mortality in very preterm infants: a birth-register study.** *Pediatrics* 2007, **119**:e257–e263.
62. Chien LY, Whyte R, Aziz K, Thiessen P, Matthew D, Lee SK: **Improved outcome of preterm infants when delivered in tertiary care centers.** *Obstet Gynecol* 2001, **98**:247–252.
63. Lui K, Abdel-Latif ME, Allgood CL, Bajuk B, Oei J, Berry A, Henderson-Smart D: **Improved outcomes of extremely premature outborn infants: effects of strategic changes in perinatal and retrieval services.** *Pediatrics* 2006, **118**:2076–2083.
64. Shah PS, Shah V, Qiu Z, Ohlsson A, Lee SK: **Improved outcomes of outborn preterm infants if admitted to perinatal centers versus freestanding pediatric hospitals.** *J Pediatr* 2005, **146**:626–631.
65. Vanpee M, Walfridsson-Schultz U, Katz-Salamon M, Zupancic JA, Pursley D, Jonsson B: **Resuscitation and ventilation strategies for extremely preterm infants: a comparison study between two neonatal centers in Boston and Stockholm.** *Acta Paediatr* 2007, **96**:10–16.
66. Tingay DG, Mills JF, Morley CJ, Pellicano A, Dargaville PA: **Trends in use and outcome of newborn infants treated with high frequency ventilation in Australia and New Zealand, 1996–2003.** *J Paediatr Child Health* 2007, **43**:160–166.
67. Roberts CL, Badgery-Parker T, Algert CS, Bowen JR, Nassar N: **Trends in use of neonatal CPAP: a population-based study.** *BMC Pediatr* 2011, **11**:89.
68. van Kaam AH, De Jaegere AP, Borensztajn D, Rimensberger PC: **Surfactant replacement therapy in preterm infants: a European survey.** *Neonatology* 2011, **100**:71–77.
69. Oh W, Poindexter BB, Perritt R, Lemons JA, Bauer CR, Ehrenkranz RA, Stoll BJ, Poole K, Wright LL: **Association between fluid intake and weight loss during the first ten days of life and risk of bronchopulmonary dysplasia in extremely low birth weight infants.** *J Pediatr* 2005, **147**:786–790.
70. Kotsopoulos K, Benadiba-Torch A, Cuddy A, Shah PS: **Safety and efficacy of early amino acids in preterm <28 weeks gestation: prospective observational comparison.** *J Perinatol* 2006, **26**:749–754.
71. Lai LS, McCrindle BW: **Variation in the diagnosis and management of patent ductus arteriosus in premature infants.** *Paediatr Child Health* 1998, **3**:405–410.
72. Clerihew L, McGuire W: **Antifungal prophylaxis for very low birthweight infants: UK national survey.** *Arch Dis Child Fetal Neonatal Ed* 2008, **93**:F238–F239.

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Brief Parenteral Nutrition Accelerates Weight Gain, Head Growth Even in Healthy VLBWs

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Abstract

Introduction: Whether parenteral nutrition benefits growth of very low birth weight (VLBW) preterm infants in the setting of rapid enteral feeding advancement is unclear. Our aim was to examine this issue using data from Japan, where enteral feeding typically advances at a rapid rate.

Methods: We studied 4005 hospitalized VLBW, very preterm (23–32 weeks' gestation) infants who reached full enteral feeding (100 ml/kg/day) by day 14, from 75 institutions in the Neonatal Research Network Japan (2003–2007). Main outcomes were weight gain, head growth, and extra-uterine growth restriction (EUGR, measurement $<10^{th}$ percentile for postmenstrual age) at discharge.

Results: 40% of infants received parenteral nutrition. Adjusting for maternal, infant, and institutional characteristics, infants who received parenteral nutrition had greater weight gain [0.09 standard deviation (SD), 95% CI: 0.02, 0.16] and head growth (0.16 SD, 95% CI: 0.05, 0.28); lower odds of EUGR by head circumference (OR 0.66, 95% CI: 0.49, 0.88). No statistically significant difference was seen in the proportion of infants with EUGR at discharge. SGA infants and infants who took more than a week until full feeding had larger estimates.

Discussion: Even in infants who are able to establish enteral nutrition within 2 weeks, deprivation of parenteral nutrition in the first weeks of life could lead to under nutrition, but infants who reached full feeding within one week benefit least. It is important to predict which infants are likely or not likely to advance on enteral feedings within a week and balance enteral and parenteral nutrition for these infants.

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Introduction

Guidelines for nutritional support of very low birth weight (VLBW, <1500 grams) and very preterm (<32 completed weeks' gestation) infants often emphasize parenteral nutrition as the main source of nutrition for the first weeks of life [1,2]. However, some infants are able to advance enteral nutrition quickly, particularly infants fed breast milk [3–6]. Additionally, providing parenteral nutrition has risks, such as infection, thrombosis, and other complications associated with central venous access [7], and cholestasis [8], and also costs more than feeding enterally. Given the important benefits of early enteral feeding [9], as well as the risks and cost inherent to providing parenteral nutrition, it is important to quantify the benefit of parenteral nutrition in the context of rapid advancement of enteral nutrition. In other words, it is important to clarify the extent to which infants who reach full enteral nutrition at a rapid rate also benefit from parenteral nutrition. This information would inform guidelines about the routine vs. selective parental nutrition use for VLBW preterm infants, particularly those infants expected to achieve full enteral feeding at a rapid rate.

The aim of this study was to quantify the growth benefit of parenteral nutrition in VLBW preterm infants who are able to advance enteral feedings rapidly. Our analysis capitalizes on the Japanese experience in which advancement of enteral feeding in the neonatal intensive care unit (NICU) is typically more rapid than in other countries, possibly due to the high rate of breast milk feeding for preterm infants and the low background necrotizing enterocolitis (NEC) rate [10,11]. Historically the rate of NEC is below 1% in VLBW infants in the Japanese Neonatal Research Network, as compared with 7–10% in the U.S. or other populations [11–13]. We hypothesized that despite routine rapid enteral feeding advancement, parenteral nutrition would nonetheless be beneficial to growth.

Methods

Design, Setting, and Participants

We used data from the Neonatal Research Network of Japan (NRN), a multi-center registry of VLBW infants cared for in 75 participating level III perinatal centers in Japan and funded by a grant from the Ministry of Health, Labor and Welfare in 2004. All registered hospitals provided individual patient data including obstetric, delivery, in-hospital care, and follow-up data at age 1.5 and 3 years to the central committee. The central committee identified potential data errors and requested that individual institutions correct data by going back to medical charts when needed. A description of the NRN cohort including morbidities, mortalities, and outcomes at age 1.5 and 3 years, has been reported previously [14–16].

Figure 1 outlines the population used for this analysis. The NRN cohort included 16001 VLBW infants who were hospitalized in 75level III NICU's in Japan from January 2003 to December 2007. Of these, 9152 were born at 24–32 weeks of gestation. We excluded infants with major congenital anomalies (n = 477) and infants admitted to the NICU more than 24 hours after birth (n = 126). In the remaining 8549 infants, 61% of the infants reached 100 ml/kg/day full feeding within 14 days, and 16% achieved this within 7 days. As our interest was mainly in the effect of parenteral nutrition on growth in infants who reached full enteral nutrition fairly rapidly, we restricted our analysis to infants who reached full feeding within 14 days (n = 5270), of whom 41% (n = 1784) had received parenteral nutrition during admission. We further excluded infants who died before discharge (n = 39), infants discharged after 48 weeks corrected age (n = 348); infants who developed NEC (n = 13) or underwent surgery (n = 385); and infants missing growth data (n = 108). We also excluded 344 subjects due to missing covariate data. Thus our total sample size for analysis was 4005.

Definition of Diseases and Outcomes

Our main exposure was any use of parenteral nutrition during the hospital stay, which the hospitals provided as yes, or no for each subject. No subject was missing this information.

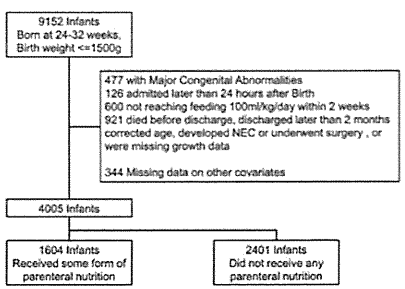

Figure 1. Population Flow Chart.
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Our main outcome was extra-uterine weight gain and head growth, which we defined as the change in SD score of each measurement from birth to discharge. These SD scores were calculated using sex, parity, and gestational length (by day) specific growth references for birth weight [17] and head circumference, obtained from vaginal deliveries during 2003–2005 in Japan. We also classified infants as being small for gestational age (SGA) or having extra-uterine growth restriction (EUGR), both proxies for intra-uterine and extra-uterine growth. SGA was defined as birth weight being under the 10^{th} percentile of the reference; EUGR was defined as weight and head circumference at less than the 10^{th} percentile at a given postmenstrual age, as compared with infants born at the same gestational age in the reference.

As growth can be affected by different obstetric and pediatric characteristics as well as the wellbeing of the infant, we considered as covariates the following: maternal age, parity, gestational diabetes (GDM), pregnancy induced hypertension (PIH), use of antenatal steroid, multiplicity, route of delivery, gestational length, sex, SD scores of weight and head circumference at birth, Apgar score at 5 minutes, use of mechanical ventilation, diagnosis and stage of intra-ventricular hemorrhage (IVH), diagnosis of bronchopulmonary dysplasia (BPD), periventricular hemorrhage (PVL), and days taken to reach full enteral feeding.

In all analyses we categorized these variables as follows: maternal age (14–20, 20–34, 35–50 years), parity (0, 1, 2 and above) multiplicity (singleton, twin, triplets or more), route of delivery (cesarean or vaginal), Apgar score at 5 minutes (0–4, 5–10), use of ventilation (no ventilation, ventilation for under 7 days, ventilation over 7 days), IVH (no IVH, grade 1–2 IVH, grade 3–4 IVH), and each completed week of gestation at birth (24 to 32 weeks).

Statistical Analysis

First, we compared maternal and infant characteristics between infants who received parenteral nutrition (n = 1604) and those who did not (n = 2401). Next, as extra-uterine growth is affected by both gestational age and intrauterine growth, we stratified our sample by each week of gestation and intrauterine growth status (SGA or not) and examined each of the following across strata as well as by use of parenteral nutrition or not: change in SD scores

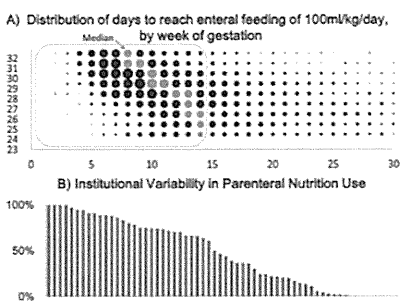

Figure 2. Comparison of nutritional practices in 75 institutions in Japan. A) Distribution of days to reach 100mlperkgperday of milk in 8,549 very low birth weight infants (23–32 weeks). B) Variability in usage of parenteral nutrition in very low birth weight infants (23–32 weeks) who reached full enteral feeding within 2 weeks.
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Table 1. Maternal and infant characteristics of 4,005 very low birth weight infants of 24–32 weeks' of gestation who reached full enteral feeding within 2 weeks.

		Infants who did not receive parenteral nutrition (n = 2401)	Infants who received parenteral nutrition (n = 1604)
		Mean (SD) or percentage	
Maternal Characteristics			
Maternal age**		30.6 (5.1)	31.2(5.1)
Number of previous deliveries		0.7(0.8)	0.6(0.8)
Number of fetuses		1.3(0.6)	1.3(0.6)
Gestational diabetes(%)		1.6%	1.8%
Pregnancy induced hypertension(%)		19.3%	19.5%
Use of antenatal steroids(%)**		42.2%	48.8%
Cesarean section (%)		77.3%	78.0%
Infant Characteristics			
Gestational length (weeks)**		29.7 (2.0)	28.7 (2.2)
Length of stay (days)**		75.6 (25.2)	84.2 (26.8)
Apgar score at 5 minutes**		8.1 (1.4)	7.7 (1.7)
Days to reach 100 ml per kg per day enteral feeding**		8.9 (2.7)	10.3 (2.6)
Birth Weight (grams)		1176 (234)	1053 (257)
Weight for gestational age, at birth (SD)		−0.90(1.0)	−0.94 (1.2)
Birth Head Circumference (cms)**		26.6 (2.0)	25.7 (2.2)
Head Circumference for gestational age, at birth (SD)		−0.20 (0.8)	−0.21 (0.8)
Weight at discharge (grams)**		2649 (452)	2713 (497)
Head Circumference at Discharge (cms)		34.2 (1.8)	34.3 (1.8)
Male (%)		49.8%	51.8%
Mechanical ventilation**	No use (%)	24.2%	24.2%
	Less than 1 week (%)	33.4%	33.4%
	More than 1 week (%)	42.4%	42.4%
Intra-Ventricular Hemorrhage**	None(%)	91.0%	91.0%
	Grade 1–2 (%)	7.1%	7.1%
	Grade 3–4 (%)	1.9%	1.9%
PPHN (%)**		1.4%	2.9%
Sepsis (%)**		2.1%	3.8%
BPD (%)**		21.6%	33.4%
PVL (%)		2.8%	3.4%
EUGR by weight (%)		58%	58%
EUGR by head circumference (%)		12%	12%

Full enteral feeding: 100 ml per kg per day of milk.
*: $p<0.05$.
**: $p<0.005$.
doi:10.1371/journal.pone.0088392.t001

of weight and head circumference at admission and discharge, and length of hospitalization in days.

To account for potential confounding by maternal and infant characteristics, and to account for clustering within institutions, we performed generalized linear mixed models (logistic regression with random intercepts) to estimate the effect of parenteral nutrition on our outcomes of interest (weight gain, head growth, odds of EUGR, and days of admission). For selection of confounders we used parity, GDM, PIH, use of antenatal steroid, multiplicity, gestational length, sex, Apgar score at 5 minutes, IVH stage, use of mechanical ventilation, day of reaching full enteral feeding, as well as birth measurements, due to their clinical relevance and usage in previous papers [5,18,19].

We performed all analyses using SAS version 9.3 (SAS Institute, Cary, NC).

Ethics Statement

All information about the infants was collected anonymously, and the stored data were unlinked from individual data. Written informed consent was obtained from the parents or guardians on behalf of each child enrolled in this study before receiving any data. The protocol of this study was approved by the Central Internal Review Board at Tokyo Women's Medical University, where all data were collected and stored. The database was registered as UMIN000006961.

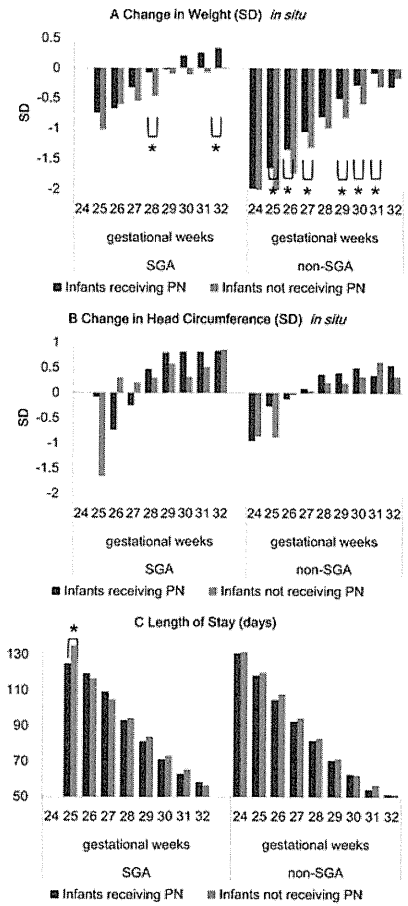

Figure 3. Comparison of infants that received and did not receive parental nutrition, stratified by gestational age and intrauterine growth. A) Change in weight (SD) *in situ*, B) Change in head circumference (SD) *in situ*, and C) Length of stay (days), of 4,005 very low birth weight infants of 24–32 weeks of gestation that reached full enteral feeding within 2 weeks. Figure legends for Figure 3: Full enteral feeding: 100 ml per kg per day of milk. PN: parenteral nutrition. SGA: small for gestational age, defined as birth weight <10th percentile for postmenstrual age.
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Results

In **Figure 2a** we show the distribution of days until enteral feeding 100 ml/kg/day (full feeding) within 8549 infants without major congenital anomalies and were admitted within 24 hours after birth, by each week of gestation. The distribution was skewed to the right, with 61% of the infants reaching full feeding within 14 days, and 16% achieving this within 7 days. Time to reach full enteral feeding was greater for infants of lower gestational age, but over 50% of infants born in each gestational week reached full feeding within 14 days. This was as expected from the customs in Japanese NICUs which prefer to initiate and increase enteral feeding as soon as possible.

In **Figure 2b**, we show the wide inter-institutional variation in usage of parenteral nutrition.

In **Table 1** we show maternal and infant characteristics for the 4005 infants who reached full enteral feeding by day 14, grouped by whether they received parenteral nutrition.

Infants who received parenteral nutrition were born earlier and were generally sicker, for example they were more likely to have needed mechanical ventilation, were more likely to have BPD, and took longer to reach full enteral feeding. Those receiving parenteral nutrition also had lower weight and smaller head circumference at birth, but showed higher weight and larger head circumference at discharge.

In **Figure 3** we show the average change in weight and head circumference SD from birth to discharge, as well as length of stay, for infants who received and did not receive parenteral nutrition, stratified by intrauterine growth (SGA and non-SGA) as well as week of gestation. Even after stratification, infants receiving parenteral nutrition showed greater growth in most categories.

Table 2 shows the estimated effect of administering parenteral nutrition on growth and length of stay, adjusted for maternal and infant characteristics listed in **Table 1**. Infants receiving parenteral nutrition showed greater weight gain and head growth: on average 0.09 (95% confidence interval [CI] 0.02, 0.16) SD greater weight gain and 0.16 (95% CI 0.05, 0.28) SD greater head growth. They also tended to have lower odds of being EUGR by weight (OR 0.85, 95% CI 0.66, 1.08) and head circumference (OR 0.66, 95% CI 0.49, 0.88) at discharge, compared to those not receiving parenteral nutrition. Length of stay was 1.29 (95% CI 0.12, 2.45) days shorter for infants who received parenteral nutrition. There was no significant association of parenteral nutrition with adverse outcomes: BPD (OR: 0.85; 95%CI 0.66–1.08); and PVL (OR: 1.19; 95%CI 0.76–1.87).

Effect modification by weeks of gestation (24–27 weeks or 28–32 weeks), intrauterine growth (SGA or non-SGA), and day to reach full feeding (0–7 days or 8–14 days) were not statistically significant, and though estimates differed slightly for each subgroup, as shown in **Figure 4**, the all effects were in the same direction.

However, point estimates of the effect of parenteral nutrition was largest in SGA infants and infants who took more than one week until full feeding, and smallest in infants who reached full feeding within a week. Point estimates of increase in weight was largest in SGA infants (0.14SD; 95%CI −0.09, 0.37) and infants reaching full feeding after one week (0.08SD; −0.01, 0.31), and smallest in infants who reached full feeding within one week (0.05SD; −0.22, 0.32). Similarly, point estimates of increase in head circumference was largest in SGA infants (0.35SD; −0.03, 0.73) and smallest in non-SGA infants (0.10SD; 0.15, 0.73) and infants reaching full feeding within one week (0.08SD; −0.25, 0.41). Point estimates for effect on length of stay was also largest in SGA infants and infants born at 28 to 32 weeks of gestation, and

Table 2. Adjusted effect of parenteral nutrition use on weight, head circumference, and length of NICU stay.

	Difference (95% CI)	p-value
Growth parameters in z-scores		
Weight gain (SD)	0.09 (0.02, 0.16)	0.01
Head growth (SD)	0.16 (0.05, 0.28)	0.004
Length of stay (days)	−1.29 (0.12, 2.45)	0.03
	Odds ratio (95% CI)	**p-value**
Growth parameters by Extrauterine Growth		
EUGR by weight (OR)	0.85 (0.66, 1.08)	0.18
EUGR by head circumference (OR)	0.66 (0.49, 0.88)	0.005

Analysis of 4,005 very low birth weight infants of 24–32 weeks' gestation who reached full enteral feeding within 2 weeks.
Generalized linear mixed models (logistic regression with random intercepts) used to accounting for clustering within institutions. Adjusted for selected maternal (maternal age, number of previous deliveries, number of fetuses, gestational diabetes, pregnancy induced hypertension, use of antenatal steroids, mode of delivery), and infant (gestational length, sex, birth weight, birth head circumference, Apgar score at 5 minutes, days to reach 100 ml per kg per day enteral feeding, length of stay) characteristics.
Full enteral feeding: 100 ml per kg per day of milk.
EUGR: Extra-uterine growth restriction; defined as weight or head circumference $<10^{th}$ percentile for postmenstrual age.
doi:10.1371/journal.pone.0088392.t002

nearly null in infants who reached full feeding within a week, were born under 1000 g, or were born at 24–27 weeks of gestation.

Discussion

We observed that among VLBW preterm (24–32 weeks' gestation) infants who reached full enteral feedings within 2 week of birth, those infants provided with parenteral nutrition demonstrated greater weight gain and head growth, and a lower prevalence of EUGR by weight, as compared with those who did not receive parenteral nutrition. To the best of our knowledge, this study is the first to show that parenteral nutrition promotes weight gain even in preterm infants who are able to advance enteral nutrition at a fairly fast rate.

Research to inform optimal nutritional care of the preterm infant is important because inadequate nutrition leads to poor growth extrauterine growth, which in turn has been linked with poor later neurocognitive outcomes [20–22]. EUGR is highly prevalent among VLBW and ELBW infants [20,23] and can be modified by nutritional practices in the NICU [24,25]

Historically, clinicians have been hesitant to advance enteral feedings at a rapid rate due to concerns about intestinal immaturity and the associated risk of NEC, a serious and often life-threatening complication of preterm birth. [26]. Research focused on providing earlier parenteral nutrition, such as intravenous amino acids, has shown a beneficial impact on growth of VLBW infants [27]. For example, Poindexter reported that early provision of amino acids was associated with significantly better growth at 36 weeks postmenstrual age. Similarly, Maggio reported changes in parenteral nutrition practice improved growth outcomes at discharge. Our results are consistent with both of those studies in supporting the benefit of parenteral nutrition on growth of VLBW infants.

However, in other studies of early parenteral nutrition and growth, enteral feedings were advanced slowly. In the Poindexter study, subjects took on average 32–34 days to reach 110 kcal/kg/day enteral feeding, and were administered parenteral nutrition for on average 32 days [28]. In the Maggio study, subjects took on average 24–27 days to reach 150 ml/kg/day enteral feeding, and were administered parenteral nutrition for 24–27 days [27]. Even the recent studies shown in a meta-analysis by Moyses [9] on the effect of parenteral nutrtion on growth, show that time to full feeds took an average of 15 to 33 days [29–33]. In contrast, in our study population, enteral feedings were advanced much more quickly, on average over 8–10 days. To our knowledge, no study prior to ours has evaluated the impact of parenteral nutrition in the setting of more rapid advance of enteral feedings.

Additionally, through subgroup analysis we found that overall, SGA infants and infants reaching full feeding after one week seemed to benefit most from parenteral nutrition in means of growth and shorter stay. Infants who reached full feeding within one week seemed to benefit least. Our findings suggest that it is important to predict which infants are likely or not likely to advance on enteral feedings within a week.

Our study has several limitations. First, we did not have data on the timing or duration of parenteral nutrition. Therefore we had to exclude infants who would be receiving parenteral nutrition due to their complications: infants that had NEC, surgery, or died during hospitalization, and thus we could not set these conditions as outcomes. However, we were most interested in the effects of parenteral nutrition in otherwise healthy preterm infants. Second, although a number of maternal and child characteristics were available, residual confounding may occur by characteristics of infants who received parenteral nutrition that could promote their growth, for instance other differences in nutritional practice. Finally, our study was conducted in Japan where neonatal intensive care practices differ from the U.S. and other countries. However, our setting provides a unique opportunity to examine the role of parenteral nutrition for infants who advance quickly on enteral nutrition, and we believe our findings will be relevant for non-Japanese populations of VLBW infants as well.

In summary, our results support the use of parenteral nutrition to improve weight gain and head growth, even among relatively healthy VLBW infants who reach full enteral feeding within 2 weeks. For infants reaching full enteral feedings in 1 week or less, the benefit was smaller. These findings will be useful for clinicians weighing the risks and benefits of providing parenteral nutrition to very low birth weight infants, particularly those who are expected to advance enteral nutrition rapidly.

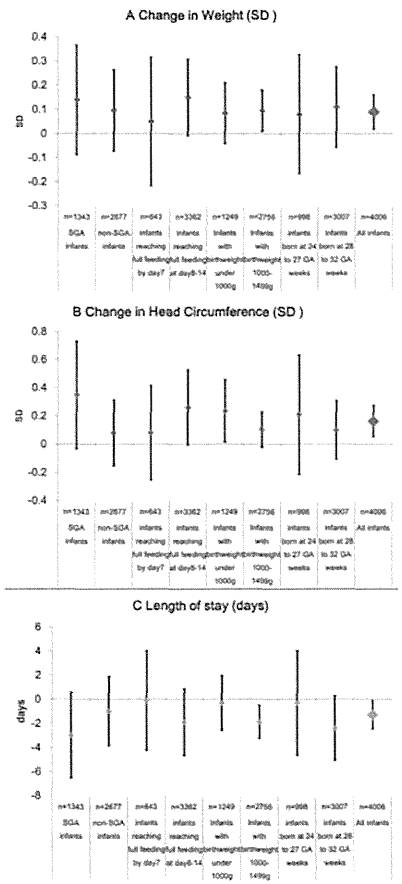

Figure 4. Estimated effect of administering parenteral nutrition. A) Change in weight (SD) *in situ*, B) Change in head circumference (SD) *in situ*, and C) Length of stay (days). Analysis of 4,005 very low birth weight infants of 24–32 weeks of gestation who reached full enteral feeding within 2 weeks. Legends for Figure 4: Generalized linear mixed models (logistic regression with random intercepts) used to accounting for clustering within institutions. Adjusted for selected maternal (maternal age, number of previous deliveries, number of fetuses, gestational diabetes, pregnancy induced hypertension, use of antenatal steroids, mode of delivery), and infant (gestational length, sex, birth weight, birth head circumference, Apgar score at 5 minutes, days to reach 100 ml per kg per day enteral feeding, length of stay) characteristics. Full enteral feeding: 100 ml per kg per day of milk.
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Author Contributions

Conceived and designed the experiments: NM. Performed the experiments: NM SK MF. Analyzed the data: NM. Contributed reagents/materials/analysis tools: NM MBB MCM RM HN. Wrote the paper: NM MBB. Interpreted the data: NM MBB MCM RM. Reviewed and contributed to drafting the final manuscript: RM HN SK MF.

References

1. Ehrenkranz RA (2007) Early, aggressive nutritional management for very low birth weight infants: what is the evidence? Semin Perinatol 31: 48–55.

2. Denne SC, Poindexter BB (2007) Evidence supporting early nutritional support with parenteral amino acid infusion. Semin Perinatol 31: 56–60.
3. Kennedy KA, Tyson JE, Chamnanvanakij S (2000) Rapid versus slow rate of advancement of feedings for promoting growth and preventing necrotizing enterocolitis in parenterally fed low-birth-weight infants. Cochrane Database Syst Rev: Cd001241.
4. Bombell S, McGuire W (2009) Early trophic feeding for very low birth weight infants. Cochrane Database Syst Rev: Cd000504.
5. Ehrenkranz RA (2010) Early nutritional support and outcomes in ELBW infants. Early Hum Dev 86 Suppl 1: 21–25.
6. Uhing MR, Das UG (2009) Optimizing growth in the preterm infant. Clin Perinatol 36: 165–176.
7. Blanco CL, Falck A, Green BK, Cornell JE, Gong AK (2008) Metabolic responses to early and high protein supplementation in a randomized trial evaluating the prevention of hyperkalemia in extremely low birth weight infants. J Pediatr 153: 535–540.
8. Hughes CA, Talbot IC, Ducker DA, Harran MJ (1983) Total parenteral nutrition in infancy: effect on the liver and suggested pathogenesis. Gut 24: 241–248.
9. Moyses HE, Johnson MJ, Leaf AA, Cornelius VR (2013) Early parenteral nutrition and growth outcomes in preterm infants: a systematic review and meta-analysis. Am J Clin Nutr 97: 816–826.
10. Isayama T, Lee SK, Mori R, Kusuda S, Fujimura M, et al. (2012) Comparison of mortality and morbidity of very low birth weight infants between Canada and Japan. Pediatrics 130: e957–965.
11. Kusuda S, Fujimura M, Uchiyama A, Totsu S, Matsunami K (2012) Trends in morbidity and mortality among very-low-birth-weight infants from 2003 to 2008 in Japan. Pediatr Res 72: 531–538.
12. Horbar JD, Badger GJ, Carpenter JH, Fanaroff AA, Kilpatrick S, et al. (2002) Trends in mortality and morbidity for very low birth weight infants, 1991–1999. Pediatrics 110: 143–151.
13. Yee WH, Soraisham AS, Shah VS, Aziz K, Yoon W, et al. (2012) Incidence and timing of presentation of necrotizing enterocolitis in preterm infants. Pediatrics 129: e298–304.
14. Kono Y, Mishina J, Sato N, Watanabe T, Honma Y (2008) Developmental characteristics of very low-birthweight infants at 18 months' corrected age according to birthweight. Pediatr Int 50: 23–28.
15. Kono Y, Mishina J, Yonemoto N, Kusuda S, Fujimura M (2011) Outcomes of very-low-birthweight infants at 3 years of age born in 2003–2004 in Japan. Pediatr Int 53: 1051–1058.
16. Kusuda S, Fujimura M, Sakuma I, Aotani H, Kabe K, et al. (2006) Morbidity and mortality of infants with very low birth weight in Japan: center variation. Pediatrics 118: e1130–1138.
17. Uehara R, Miura F, Itabashi K, Fujimura M, Nakamura Y (2011) Distribution of birth weight for gestational age in Japanese infants delivered by cesarean section. J Epidemiol 21: 217–222.
18. Belfort MB, Rifas-Shiman SL, Sullivan T, Collins CT, McPhee AJ, et al. (2011) Infant growth before and after term: effects on neurodevelopment in preterm infants. Pediatrics 128: e899–906.
19. Martin CR, Brown YF, Ehrenkranz RA, O'Shea TM, Allred EN, et al. (2009) Nutritional practices and growth velocity in the first month of life in extremely premature infants. Pediatrics 124: 649–657.
20. Ehrenkranz RA, Dusick AM, Vohr BR, Wright LL, Wrage LA, et al. (2006) Growth in the neonatal intensive care unit influences neurodevelopmental and growth outcomes of extremely low birth weight infants. Pediatrics 117: 1253–1261.
21. Ehrenkranz RA, Das A, Wrage LA, Poindexter BB, Higgins RD, et al. (2011) Early nutrition mediates the influence of severity of illness on extremely LBW infants. Pediatr Res 69: 522–529.
22. Belfort MB, Gillman MW (2013) Healthy infant growth: what are the trade-offs in the developed world? Nestle Nutr Inst Workshop Ser 71: 171–184.
23. Dusick AM, Poindexter BB, Ehrenkranz RA, Lemons JA (2003) Growth failure in the preterm infant: can we catch up? Semin Perinatol 27: 302–310.
24. Stephens BE, Walden RV, Gargus RA, Tucker R, McKinley L, et al. (2009) First-week protein and energy intakes are associated with 18-month developmental outcomes in extremely low birth weight infants. Pediatrics 123: 1337–1343.
25. Dinerstein A, Nieto RM, Solana CL, Perez GP, Otheguy LE, et al. (2006) Early and aggressive nutritional strategy (parenteral and enteral) decreases postnatal growth failure in very low birth weight infants. J Perinatol 26: 436–442.
26. Higgins RD, Devaskar S, Hay WW Jr, Ehrenkranz RA, Greer FR, et al. (2012) Executive summary of the workshop "Nutritional Challenges in the High Risk Infant". J Pediatr 160: 511–516.
27. Maggio L, Cota F, Gallini F, Lauriola V, Zecca C, et al. (2007) Effects of high versus standard early protein intake on growth of extremely low birth weight infants. J Pediatr Gastroenterol Nutr 44: 124–129.
28. Poindexter BB, Langer JC, Dusick AM, Ehrenkranz RA (2006) Early provision of parenteral amino acids in extremely low birth weight infants: relation to growth and neurodevelopmental outcome. J Pediatr 148: 300–305.
29. Aroor AR, Krishnan L, Reyes Z, Fazallulah M, Ahmed M, et al. (2012) Early versus Late Parenteral Nutrition in Very Low Birthweight Neonates: A retrospective study from Oman. Sultan Qaboos Univ Med J 12: 33–40.
30. Garcia LV, Erroz IO, Freire MM, Munuzuri AP, Souto AB, et al. (2012) [Does early parenteral protein intake improve extrauterine growth in low birth weight preterms?]. An Pediatr (Barc) 76: 127–132.
31. Janeiro P, Cunha M, Marques A, Moura M, Barroso R, et al. (2010) Caloric intake and weight gain in a neonatal intensive care unit. Eur J Pediatr 169: 99–105.
32. Trintis J, Donohue P, Aucott S (2010) Outcomes of early parenteral nutrition for premature infants. J Perinatol 30: 403–407.
33. Blanco CL, Gong AK, Schoolfield J, Green BK, Daniels W, et al. (2012) Impact of early and high amino acid supplementation on ELBW infants at 2 years. J Pediatr Gastroenterol Nutr 54: 601–607.

PLOS ONE

Expression Level and Subcellular Localization of Heme Oxygenase-1 Modulates Its Cytoprotective Properties in Response to Lung Injury: A Mouse Model

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Abstract

Premature infants exposed to hyperoxia suffer acute and long-term pulmonary consequences. Nevertheless, neonates survive hyperoxia better than adults. The factors contributing to neonatal hyperoxic tolerance are not fully elucidated. In contrast to adults, heme oxygenase (HO)-1, an endoplasmic reticulum (ER)-anchored protein, is abundant in the neonatal lung but is not inducible in response to hyperoxia. The latter may be important, because very high levels of HO-1 overexpression are associated with significant oxygen cytotoxicity in vitro. Also, in contrast to adults, HO-1 localizes to the nucleus in neonatal mice exposed to hyperoxia. To understand the mechanisms by which HO-1 expression levels and subcellular localization contribute to hyperoxic tolerance in neonates, lung-specific transgenic mice expressing high or low levels of full-length HO-1 (cytoplasmic, HO-1-FL(H) or HO-1-FL(L)) or C-terminally truncated HO-1 (nuclear, Nuc-HO-1-TR) were generated. In HO-1-FL(L), the lungs had a normal alveolar appearance and lesser oxidative damage after hyperoxic exposure. In contrast, in HO-1-FL(H), alveolar wall thickness with type II cell hyperproliferation was observed as well worsened pulmonary function and evidence of abnormal lung cell hyperproliferation in recovery from hyperoxia. In Nuc-HO-1-TR, the lungs had increased DNA oxidative damage, increased poly (ADP-ribose) polymerase (PARP) protein expression, and reduced poly (ADP-ribose) (PAR) hydrolysis as well as reduced pulmonary function in recovery from hyperoxia. These data indicate that low cytoplasmic HO-1 levels protect against hyperoxia-induced lung injury by attenuating oxidative stress, whereas high cytoplasmic HO-1 levels worsen lung injury by increasing proliferation and decreasing apoptosis of alveolar type II cells. Enhanced lung nuclear HO-1 levels impaired recovery from hyperoxic lung injury by disabling PAR-dependent regulation of DNA repair. Lastly both high cytoplasmic and nuclear expression of HO-1 predisposed to long-term abnormal lung cellular proliferation. To maximize HO-1 cytoprotective effects, therapeutic strategies must account for the specific effects of its subcellular localization and expression levels.

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Introduction

Premature neonates with altered lung function are exposed to hyperoxia to maintain adequate oxygenation but they may develop bronchopulmonary dysplasia, a pulmonary disease with long-term sequelae including neurodevelopmental delay [1–3]. Interestingly, humans, in particular neonates, have developed some adaptive mechanisms to mitigate oxidative stress. Although prolonged hyperoxic exposure causes injury to the neonatal lung, neonatal rodents are more tolerant to hyperoxia than their adult counterparts [4]. Some have implicated increased antioxidant enzyme activity and a reduced superoxide-generating capacity in the neonatal lung [5–7]. Nevertheless, these observations do not fully explain the enhanced hyperoxic tolerance in neonates. One molecule with antioxidant properties, HO-1, the rate-limiting enzyme in the degradation of heme, is robustly inducible in oxidative stress such as hyperoxia in adults [8] but differentially regulated in neonatal rodents as compared to the adult [9]. Despite increased HO-1 expression in the perinatal period, there was no difference in lung HO-1 mRNA levels in newborn rats (< 12 hours old) exposed to hyperoxia, for 3 days compared to air exposed controls, in contrast with adult models.

Whereas HO-1 knockout mice which have a shorter life span, reduced stress defenses, and disrupted postnatal lung development [10,11], lung-specific neonatal HO-1 transgenic mice demonstrated vasculoprotective effects in hyperoxia [12]. However, very high levels of HO-1 expression were associated with significant oxygen cytotoxicity *in vitro*, suggesting there is a beneficial threshold of HO-1 overexpression [13]. Whether this is the case *in vivo* is not known. The HO-1 protein is anchored to the ER through a transmembrane segment located at the C-terminus [14], with the remainder in the cytoplasm [15]. Nuclear localization of HO-1 has been demonstrated in many situations including in astroglial cells during differentiation [16], in fetal lung cells under hyperoxia [17], and in brown adipocytes [18]. Interestingly, nuclear HO-1 was implicated as a regulator of DNA repair activities important to carcinogenesis [19,20] and tumor progression [21]. We have shown that HO-1 can be proteolytically cleaved from the ER to allow nuclear translocation with hypoxia [22]. This may serve to upregulate cytoprotective genes against oxidative stress [22]. Nuclear HO-1 is found in higher abundance in the lungs from neonatal mice exposed to hyperoxia compared to similarly exposed adults [23]. Could this translocation of cytoplasmic HO-1 to the nucleus contribute to tolerance against oxidative stress and promote or protect against abnormal cell proliferation and tumorigenesis *in vivo*?

To better understand the impact of HO-1 abundance and subcellular localization on hyperoxic injury and repair, lung-specific HO-1 transgenic mice were developed to express either low or high levels of cytoplasmic HO-1 or enhanced nuclear HO-1.

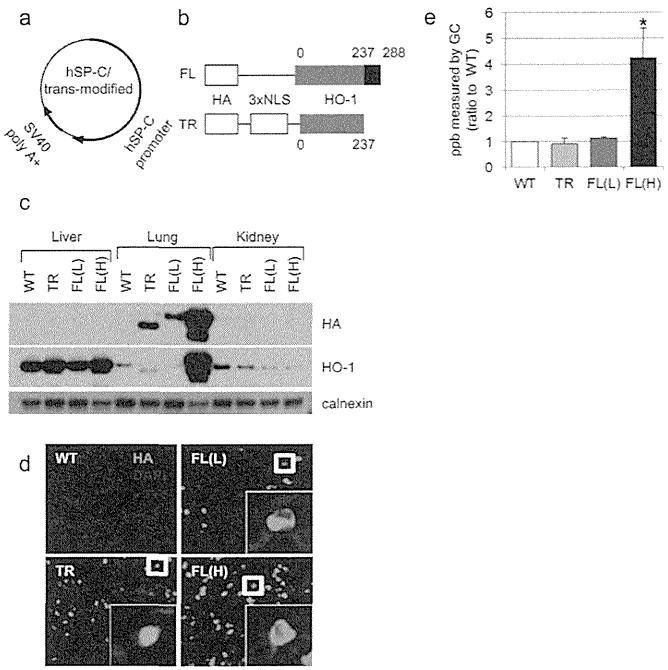

Figure 1. Expression, localization, and HO activity of HA-tagged HO-1 protein in the murine lung. (**a**) Human SP-C/trans-modified vector construct. (**b**) HA-tagged HO-1 cDNA constructs. Numbers denote amino acids in the fragment starting from the N terminus. NLS, nuclear localization sequence. (**c**) Immunoblots showing lung-specific overexpression of high or low levels of HA-tagged HO-1-FL (FL(H) or FL(L)) or Nuc-HO-1-TR (TR). Membranes were re-probed with calnexin antibodies as a loading control. (**d**) HA immunostaining (green) and nuclear DAPI staining (blue) in lung slices from HO-1-FL (FL) and Nuc-HO-1-TR (TR) transgenic mice. (**e**) Total lung HO activity in whole lung homogenates on day 14 of life. Values are the mean ± SEM of 3 separate determinations in each group. *, $p<0.05$ vs WT.
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Materials and Methods

HO-1 Transgenic Lines

All procedures and protocols were reviewed and approved by the Children's Hospital of Philadelphia's Institutional Animal Care and Use Committee in accordance with the Animal Welfare Act of the NIH. The human surfactant protein (SP)-C driven hemagglutinin (HA)-tagged HO-1-FL and C-terminal 53 amino acid Nuc-HO-1-TR cDNAs were independently engineered (Fig. 1a,b). Transgenic mice were generated using standard procedures of microinjection by the Transgenic Mouse Core Facility of the Children's Hospital of Philadelphia [24].

Hyperoxic Exposure and Recovery

Neonatal pups were randomly assigned to room air (normoxia) or 95% oxygen (hyperoxia). Exposure to hyperoxia was conducted for 72 hours in a chamber (BioSpherix, Redfield, NY), which allows for continuous monitoring and regulation of oxygen and

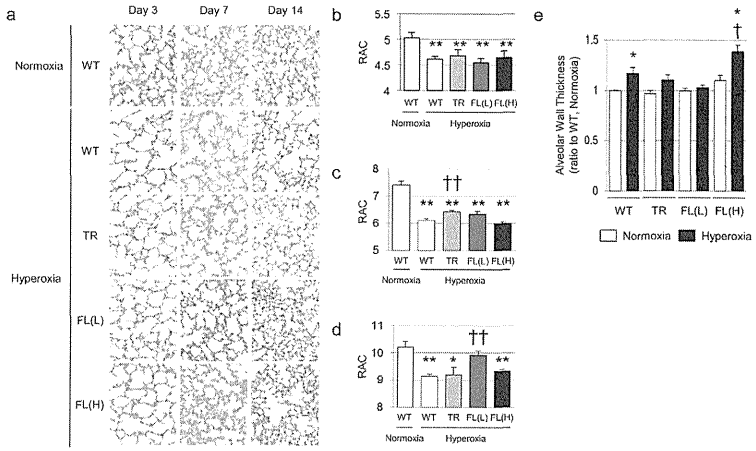

Figure 2. Morphological analysis of HO-1 trangenic mouse lungs after neonatal hyperoxic exposure and recovery in room air. (a) Hematoxylin and eosin (H&E) stained histological sections from animals on days 3, 7, and 14. (**b–d**) RAC on Days 3, 7, and 14. Each group had a minimum of 6 samples, and data are the mean ± SEM. *, p<0.05 vs normoxia **, p<0.01 vs normoxia; ††, p<0.01 vs WT/hyperoxia. (**e**) Alveolar wall thickness on day 7. Values from WT control (WT, normoxia) were set at 1 to calculate the relative values in other experimental groups. Each group had a minimum of 5 samples, and data are the mean ± SEM. *, p<0.05 vs normoxia; †, p<0.05 vs WT/hyperoxia.
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carbon dioxide. Dams were switched every 24 h between normoxia and hyperoxia. Some mice were allowed to recover in room air to adulthood (8 months of age).

Lung Tissue Collection

Mice were anesthetized with an intraperitoneal injection of ketamine hydrochloride (100 mg/kg) and xylazine hydrochloride (10 mg/kg). After the pulmonary artery was perfused with phosphate buffered saline (PBS), the right lung was excised and snap-frozen with liquid nitrogen for protein analysis. The left lung was inflated and fixed with 10% neutral-buffered formalin (HT5014, Sigma-Aldrich, St Louis, MO) for 24 hours. Lung tissue was paraffin-embedded and 5-µm thick sections were mounted on glass slides.

HO Activity Assay

Carbon monoxide production was measured as a marker of HO activity using gas chromatography as previously described [25].

Radial Alveolar Counts (RAC) and Alveolar Wall Thickness

Alveolarization was quantified by RAC, as described [26,27]. The alveolar wall thickness was measured. One lung section from 5 separate animals per study group was viewed at ×20 magnification under horizontal lines. Thickness of each septum was measured parallel to the intersecting line utilizing ImageJ software (National Institutes of Health, Bethesda, MD).

Detection of Protein Carbonylation

The detection of protein carbonylation was performed using a Protein Oxidation Detection Kit (Millipore, Billerica, MA), according to the manufacturer's instructions.

DNA Laddering Assay

Genomic DNA was isolated from frozen lung tissue using the BDtract genomic DNA isolation kit (Maxim Biotech, San Francisco, CA). Fragmented DNA was ligated to adaptor DNA fragments and subjected to PCR for amplification of cleaved genomic DNA according to the manufacturer's instructions (PCR kit for DNA ladder assay, Maxim Biotech, San Francisco, CA). Samples were run on 2% agarose-ethidium bromide gel and visualized using a UV imager.

Evaluation of Protein Levels in Lung Homogenates

Western analysis was performed to evaluate protein levels, as described [28]. The antibodies were as follows: anti-HA (clone 16B12, Covance, Richmond, CA), anti-HO-1 (SPA-896, Enzo Life Science, Plymouth Meeting, PA), anti-PARP (Cell Signaling Technology, Danvers, MA), anti-PAR (10H, Enzo Life Sciences International, Plymouth Meeting, PA), anti-PAR glycohydrolase (PARG) (Abgent, San Diego, CA), anti-lamin B (sc-6216, Santa Cruz Biochemistry, Santa Cruz, CA), and anti-calnexin (SPA-860, Stressgen, Victoria, BC, Canada), anti-phospho-p44/42 mitogen-activated protein kinase (MAPK) (extracellular signal-regulated kinase (ERK) 1/2) (Cell Signaling Technology, Danvers, MA), anti-ERK1 (Santa Cruz Biochemistry, Santa Cruz, CA), and anti-epidermal growth factor receptor (EGFR) (Cell Signaling Technology, Danvers, MA). Prior to western analysis of PAR, PARG enzyme (Trevigen, Gaithersburg, MD) was added to lung homogenate of mice and incubated at 37°C for 1 h.

Immunohistochemistry

Paraffin-embedded tissue sections were processed for indirect immunofluorescence staining or a modified method using biotin

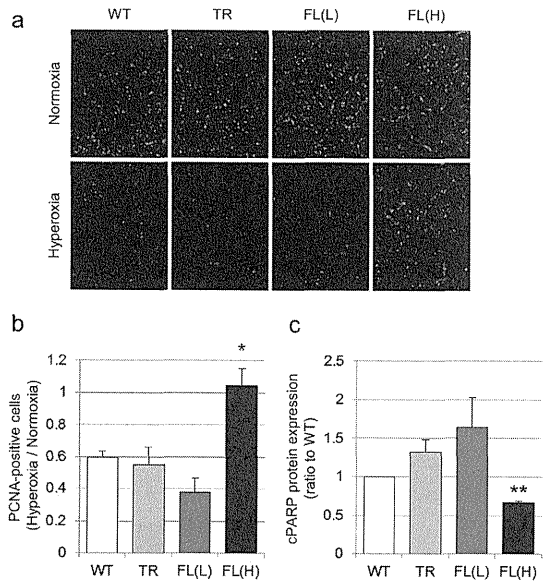

Figure 3. Evaluation of cell proliferation and apoptosis in the HO-1 transgenics exposed to neonatal hyperoxia. (a) PCNA (green) immunostaining on Day 3. (b) Quantification of PCNA immunopositive cells. Five high powered fields were counted in each lung. Each group had 3 samples, and values are expressed as a ratio to air and are the mean ± SEM. *, p<0.05 vs WT. (c) Quantification of cleaved PARP protein levels in HO-1 transgenic mice exposed to 3-day hyperoxia on Day 7. Values are the mean ± SEM of 3 determinations in each group. **, p<0.01 vs WT.
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amplification and a commercial kit (Tyramide Signal Amplification System, PerkinElmer, Waltham, MA). Sections were incubated overnight at 4°C with anti-HA (clone 16B12; Covance, Richmond CA), biotinylated anti- proliferating cell nuclear antigen (PCNA) (PC10: ab29, Abcam, Cambridge, UK), anti-pro-SP-C (AB3786, Millipore, Temecula, CA), anti-vimentin (Clone LN-6, Sigma-Aldrich, Saint Louis, MO), anti-alpha-smooth muscle actin (α-SMA; clone 1A4, Sigma-Aldrich, Saint Louis, MO), and anti-CD45 (clone 30-F11, BD Biosciences, San Diego, CA).

Immunoprecipitation

Nuclear extracts from the WT or Nuc-HO-1-TR lung were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Pierce, Rockford, IL). Immunoprecipitation was performed using anti-HA antibody conjugated to agarose beads (HA Tag IP/Co-IP kit, Pierce, Rockford IL). Briefly, tissue lysates were transferred to spin columns and anti-HA agarose beads were added followed by overnight incubation at 4°C. The columns were spun to remove the tissue lysate, and the beads were washed. The immunoprecipitated proteins were eluted and western blotting was performed.

Assessment of Respiratory Mechanics

Eight-week-old mice were anesthetized, tracheostomized and connected via the endotracheal cannula to a flexiVent system (SCIREQ Inc., Montreal, Canada). Inspiratory capacity, resistance, compliance, elastance, tissue damping, and tissue elastance were calculated.

Magnetic Resonance Imaging (MRI)

MRI was performed on 4-week-old mice. MRI images were obtained with a 7 Tesla ClinScan animal scanner (Bruker, Ettlingen, Germany) running the Syngo acquisition software (Simens, Malvern, PA). The lung was outlined in all coronal planes with a computer-assisted free-outline technique on images and the volume was calculated with the Vitrea Enterprise SuiteTM software (Vital Image, Minnetonka, MN).

Chemotactic Invasion of HO-1 Stably Infected HO-1 Null Mouse Embryonic Fibroblast (MEF) Cells

A 0.5% solution of low–melting point agarose (Invitrogen, Carlsbad, CA) was made by boiling with PBS. The solution was cooled to 40°C, and mixed with either PBS alone or epidermal growth factor (EGF) being in a solution of 0.09 µg/100 µl. Ten-microliter spots of agarose containing EGF were spotted onto the slides and allowed to cool and 3×10^6 cells were seeded onto the plates containing the slides in the presence of media with 10% fetal calf serum (FCS) and allowed to adhere for 4 h. Cells were transferred into cell culture media with 0.1% FCS and incubated for 7

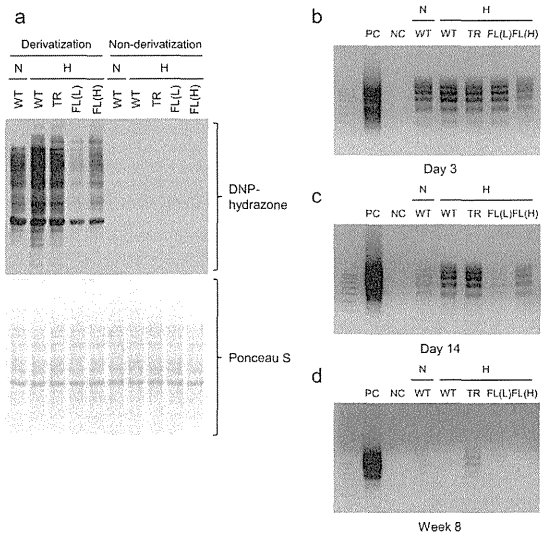

Figure 4. Oxidative stress status of protein and DNA after hyperoxic exposure. (**a**) Evaluation of dinitrophenol (DNP) immunoreactive signal in the HO-1 transgenic lungs after neonatal hyperoxic exposure. Non-derivatized proteins were loaded as negative controls. Membranes were stained with Ponceau S as a loading control. Representative image of DNA laddering in lung homogenates from 3 day old (**b**), 14 days old (**c**), and 8 week old (**d**) HO-1 transgenics exposed to hyperoxia as neonates.
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hours at 37°C. The slides were then viewed under confocal microscope.

Statistical Analysis

Values were the mean ± standard error of the mean (SEM) of separate experiments. For comparison between treatment groups, the null hypothesis that there is no difference between treatment means was tested by unpaired t-test for two groups. A p value < 0.05 was considered significant.

Results

Expression and Localization of HA-tagged HO-1 in the Lung

Three transgenic lines overexpressing HO-1-FL(H), HO-1-FL(L), or Nuc-HO-1-TR were further characterized (Fig. 1c). Fluorescence microscopy verified cytoplasmic and nuclear HA staining of alveolar epithelial cells in the HO-1-FL and Nuc-HO-1-TR, respectively (Fig. 1d).

Determination of the Enzymatic Activity of Lung HO-1

Total HO activity in HO-1-FL(H) was 4 times higher than that in wild-type (WT). HO activity was no different in the Nuc-HO-1-TR and HO-1-FL(L) compared with WT despite enhanced (2 fold increased) HO-1 protein (Fig. 1e).

Alveolar Development is Impaired after Hyperoxia and Recovery

In air, the WT developed well-organized terminal airways. In contrast, exposure of WT newborn mice to 3-day hyperoxia impaired alveolar development, resulting in alveolar simplification (Fig. 2a) and RAC were significantly reduced (Fig. 2b). Nuc-HO-1-TR, HO-1-FL(L), and HO-1-FL(H) showed reduced RAC after 3 days of hyperoxia (Fig. 2b). When WT were exposed to hyperoxia for 3 days then allowed to recover in room air for 11 days, they still had significantly decreased RAC, compared to their normoxic counterparts (Fig. 2b–d), whereas, despite worsened RAC after acute hyperoxia, the HO-1-FL(L) had significantly improved RAC after room air recovery compared with the similarly exposed WT (Fig. 2d). In contrast, neither the Nuc-HO-1-TR or the HO-1-FL(H) showed improved RAC after an 11-day recovery.

HO-1-FL(H) Exhibit Thickened Alveolar Walls with Hypercellularity when Recovering from Hyperoxic Injury

Hyperoxia-exposed WT exhibited a decrease in alveolar wall thickness at 3 days of age (not shown) and a subsequent increase at 7 days of age compared with the normoxia exposed controls (Fig. 2e). Similarly, HO-1-FL(L) demonstrated reduced alveolar wall thickness after hyperoxia (not shown), but did not exhibit increased wall thickness during recovery (Fig. 2e). In contrast, HO-1-FL(H) had persistently increased alveolar wall thickness with hypercellularity during hyperoxia and recovery (Fig. 2a,e).

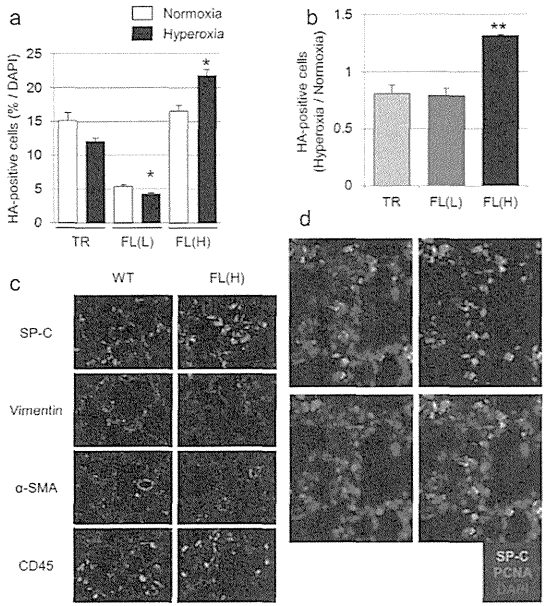

Figure 5. Evaluation of HO-1 overexpressing epithelial cells in HO-1 transgenic mice. (**a**) Quantification of HA immunopositive cells corrected for total DAPI-positive nuclei. Values are the mean ± SEM of 3 separate determinations. *, $p<0.05$ vs normoxia. (**b**) Quantification of HA immunopositive cells. as a ratio to air exposed controls. Values are the mean ± SEM of 3 separate determinations. **, $p<0.01$ vs TR, FL(L). (**c**) Immunostaining with cell-specific markers SP-C, vimentin, α-SMA, and CD45 (green), nuclei are stained with DAPI (blue). (**d**) Coimmunostaining of SP-C (green), PCNA (red) and DAPI (blue) in the HO-1-FL(H) transgenics exposed to hyperoxia as neonates.
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HO-1-FL(H) Demonstrate Increased Lung Cell Proliferation and Decreased Apoptosis after Hyperoxic Exposure

To understand whether increased alveolar wall thickness in HO-1-FL(H) was due to altered cellular proliferation and/or apoptosis, immunohistochemical staining for PCNA, a general marker of cell proliferation, and western blot analysis for cleaved PARP, a marker of apoptosis [29], were used. In WT, Nuc-HO-1-TR, and HO-1-FL(L), after hyperoxic exposure, the number of PCNA-positive cells was decreased. However, this was not the case in the HO-1-FL(H) (Fig. 3a,b). Furthermore, HO-1-FL(H) had less apoptosis after exposure to 3-day hyperoxia compared to WT (Fig. 3c).

HO-1-FL(L) have Decreased Evidence of Oxidative Injury after Hyperoxia and Recovery

Since HO-1 is an antioxidant molecule [30], we verified whether overexpression of HO-1 modulated markers of oxidative stress. As expected, increased protein carbonylation was observed in the WT after 3-day hyperoxia (Fig. 4a, lane 2), whereas HO-1-FL(L) exhibited a marked decrease in lung oxidized proteins after hyperoxic exposure compared with WT (Fig. 4a, lanes 2–5).

Adult Nuc-HO-1-TR Exposed to Hyperoxia as Neonates have Persistent DNA Damage

A known consequence of hyperoxia is DNA oxidative damage [31]. Whereas neonatal mice exposed to hyperoxia had increased lung DNA fragmentation, which persisted for up to 2 weeks (Fig. 4b,c). This resolved by adulthood in all groups (Fig. 4d) except for the Nuc-HO-1-TR, which had increased lung DNA fragmentation at 14 days of age compared with other transgenic lines and persistence into adulthood (Fig. 4c,d).

HO-1-FL(H) Exposed to Hyperoxia as Neonates have Thickened Alveolar Walls and Abnormal Alveolar Type II Cell Proliferation

The increased cell proliferation and decreased apoptosis in the lung after hyperoxia could explain the hypercellularity and thickened alveolar walls seen in HO-1-FL(H). In fact, the HO-1-FL(H) had significantly increased HA-positive cells in the lung after hyperoxia compared to other groups (Fig. 5a,b) and regions of thickened alveolar walls and hypercellularity compared with WT and the other 2 transgenic lines (Fig. 2a,c). Using cell specific antibodies, we verified that there was no increase in fibroblasts,

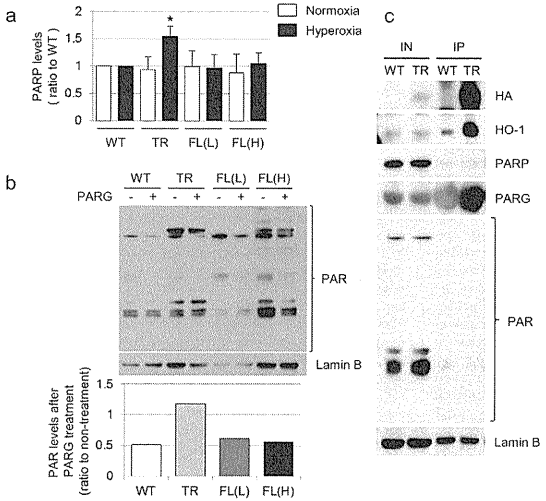

Figure 6. Assessment of lung PARP and PAR hydrolysis in 2 week old HO-1 transgenic mice exposed to hyperoxia as neonates. (**a**) Values are the mean of 3 densitometric measurements in each group. *, $p<0.05$ vs WT. (**b**) Quantification of PAR immune signal after incubation with PARG enzyme in the 14 day old HO-1-transgenic mice. Lamin B is shown as a loading control. (**c**) Immunoprecipitation of Nuc-HO-1-TR with PAR-related proteins. Representative PARG signal after immunoprecipitation with HA-tagged Nuc-HO-1-TR. Lamin B is shown as a loading control. IN, input; IP, immunoprecipitation.
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myofibroblasts, and inflammatory cells in HO-1-FL(H). However, increased numbers of alveolar type II cells were identified in the thickened alveolar walls of the HO-1-FL(H) (Fig. 5c) and co-localization of anti-pro-SP-C and anti-PCNA immunofluorescent staining was observed (Fig. 5d). In addition, the accumulation of type II cells was not diffuse but focal.

Nuc-HO-1-TR show Increased PARP and Decreased PAR Hydrolysis during Recovery from Hyperoxia

An important mediator of DNA repair is PARP. We have previously shown that PARP binds to nuclear HO-1 *in vitro* [23]. Therefore, the relationship between Nuc-HO-1-TR and PAR-related proteins was investigated. Total PARP protein levels were increased in Nuc-HO-1-TR during recovery from hyperoxia compared with WT (Fig. 6a) and PARG did not hydrolyze PAR in Nuc-HO-1-TR (Fig. 6b) in contrast to WT, HO-1-FL(L), and HO-1-FL(H). Furthermore, PARG was pulled down with HA-tagged nuclear HO-1, suggesting that nuclear HO-1 also binds PARG (Fig. 6c). This binding may limit PARG-mediated PAR hydrolysis leading to its accumulation.

Nuc-HO-1-TR and HO-1-FL(H) Exposed to Hyperoxia as Neonates have Diminished Pulmonary Function in Adulthood

Indices of pulmonary function were assessed in the mice exposed to hyperoxia as neonates at 8 weeks. In HO-1-FL(H) exposed to air, inspiratory capacity and compliance were decreased, whereas resistance, elastance, tissue elastance, and tissue damping were increased compared to WT, and exposure to hyperoxia adversely affected these parameters (Fig. 7a). Consistent with the improved RAC seen at 2 weeks in HO-1-FL(L), neonatal hyperoxic exposure did not change pulmonary function in this group. Nuc-HO-1-TR had increased compliance and decreased elastance compared to WT (Fig. 7a).

HO-1-FL(H) have Pulmonary Densities on MRI

Although exposure to hyperoxia as neonates resulted in increased total lung volume as quantified from MRI digital stacks (not shown), there were no statistical differences in total lung volume between each group. Nevertheless, the 4 week old HO-1-FL(H) exposed to 3-day hyperoxia as neonates had increased pulmonary densities on MRI (Fig. 7bc).

HO-1-FL(H) Exposed to Hyperoxia as Neonates Show Evidence of Abnormal Cell Proliferation as Adults

To understand what contributed to the pulmonary densities seen on MRI in HO-1-FL(H), we re-assessed lung histology in all groups at 8 weeks. Only the lungs of HO-1-FL(H) exposed to hyperoxia as neonates showed abnormal multinucleated intra-alveolar cells without evidence of inflammation or fibrosis (Fig. 8a). Since an association between HO-1 abundance and/or nuclear localization and tumor cell proliferation has been implied [21,32,33], we assessed the lung tissues and homogenates in all groups for markers of tumorigenesis [34] at 8 weeks. Neither

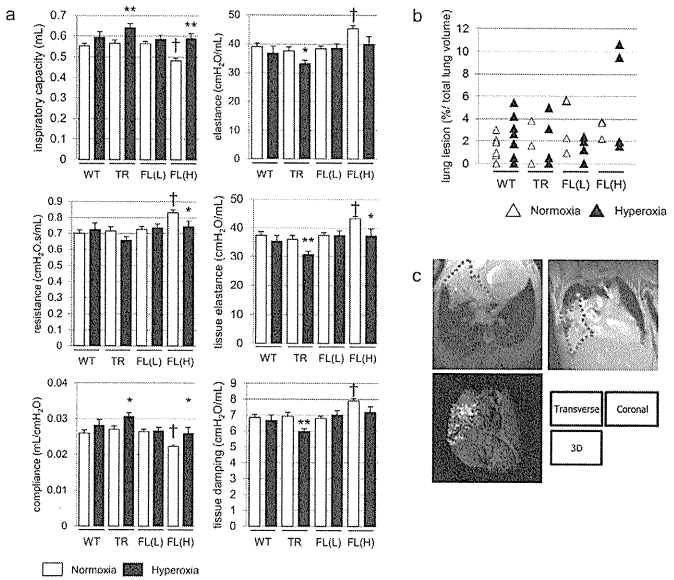

Figure 7. Pulmonary function and MRI in adulthood. (**a**) Respiratory mechanics in 8 week old HO-1 transgenic mice exposed to hyperoxia as neonates. Inspiratory capacity, resistance, compliance, elastance, tissue elastance, and tissue damping, were measured. Values are the mean ± SEM of 3 separate determinations in each group. *, $p<0.05$; ** vs normoxia; $p<0.01$ vs normoxia; †, $p<0.01$ vs WT. (**b, c**) Lung MRI in 4 day old HO-1 transgenic mice exposed to hyperoxia as neonates. (**b**) Percent ratio of lung density to total lung volume calculated using the Vitrea software after outlining the lung with a computer-assisted free-outline technique. Each value is shown as an empty (normoxia) or filled (hyperoxia) triangle. There are no statistical differences among groups. (**c**) Representative transverse, coronal, and three-dimensional reconstruction images of HO-1-FL(H) lungs exposed to 3-day hyperoxia as neonates. Dotted line outline the lung density.
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EGFR nor phosphorylated (p)-ERK immunoreactivity was elevated in WT, HO-1-FL(L), or HO-1-FL(H), but p-ERK was increased in Nuc-HO-1-TR. To further test whether localization or abundance of HO-1 promotes tumorigenesis during repair from hyperoxia, migration of HO-1 null MEF cells stably infected with HO-1-FL or Nuc-HO-1-TR cDNAs towards a solution of low-melting point agarose containing PBS with or without EGF was documented. Both HO-1-FL and Nuc-HO-1-TR MEF cells exhibited migration towards agarose with EGF although this was more evident in Nuc-HO-1-TR (Fig. 8d). In corroboration, Nuc-HO-1-TR cell had increased abundance of the EGFR (Fig. 8e). These data suggest that nuclear HO-1 promotes tumor-like behavior *in vitro*.

Discussion

Although hyperoxia is not found in nature, it is common in the clinical setting and adaptive responses exist to this challenge. Induction of HO is observed in adult rodents exposed to hyperoxia and is thought to be cytoprotective [8]. However, lung HO-1 is not induced in similarly exposed neonatal mice [9]. Furthermore, neonatal mice have increased nuclear localization of lung HO-1 in hyperoxia and adults do not [23]. We have previously demonstrated that despite beneficial effects of HO-1 at low levels of expression, there was a reversal of cytoprotection with increased HO-1 expression *in vitro* [13], indicating a beneficial threshold of HO-1 overexpression. Ours is the first report demonstrating that the degree of HO-1 overexpression and intracellular localization alters its cytoprotective abilities *in vivo*. We successfully generated two HO-1-FL transgenic mouse lines expressing HO-1 protein in the lung at low and high levels. In addition, since localization of HO-1 in the nucleus is associated with various cytoprotective effects [22], lung-specific transgenic mice expressing HO-1 protein in the nucleus were also generated. To simulate the clinical circumstance, mice were exposed to hyperoxia for 3 days then recovered in air. This model allows for an assessment of acute injury as well as repair [35].

Despite no significant increase in HO activity and similar levels of arrested alveolarization, alveolar wall thinning, and decreased alveolar cell proliferation than WT after 3 days of hyperoxia, HO-1-FL(L) recovered completely from lung injury induced by neonatal hyperoxia by 14 days. This may be due to mitigation of oxidative stress during the recovery period. The lack of long-term adverse effects on lung structure and pulmonary mechanics