

# Pigs and humans with cystic fibrosis have reduced insulin-like growth factor 1 (IGF1) levels at birth

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People with cystic fibrosis (CF) exhibit growth defects. That observation has been attributed, in part, to decreased insulin-like growth factor 1 (IGF1) levels, and the reduction has been blamed on malnutrition and pulmonary inflammation. However, patients with CF already have a reduced weight at birth, a manifestation not likely secondary to poor nutrition or inflammation. We found that, like humans, CF pigs were smaller than non-CF littermates and had lower IGF1 levels. To better understand the basis of IGF1 reduction, we studied newborn pigs and found low IGF1 levels within 12 h of birth. Moreover, humerus length and bone mineral content were decreased, consistent with less IGF1 activity in utero. These findings led us to test newborn humans with CF, and we found that they also had reduced IGF1 levels. Discovering lower IGF1 levels in newborn pigs and humans indicates that the decrease is not solely a consequence of malnutrition or pulmonary inflammation and that loss of cystic fibrosis transmembrane conductance regulator function has a more direct effect. Consistent with this hypothesis, we discovered reduced growth hormone release in organotypic pituitary slice cultures of newborn CF pigs. These findings may explain the long-standing observation that CF newborns are smaller than non-CF babies and why some patients with good clinical status fail to reach their growth potential. The results also suggest that measuring IGF1 levels might be of value as a biomarker to predict disease severity or the response to therapeutics. Finally, they raise the possibility that IGF1 supplementation beginning in infancy might be beneficial in CF.

Patients with cystic fibrosis (CF) have well-documented growth defects (1–4). However, the causes of growth defects in people with CF are still being elucidated. Malnutrition and the consequences of chronic lung infection likely play important roles (for a review, see ref. 5). However, evidence suggests that even individuals with good clinical status do not reach their full growth potential (3). An appealing candidate for explaining this discrepancy is the somatotrophic axis. Indeed, more than four decades ago, Green et al. reported that patients with CF had altered regulation of growth hormone (GH) (6). Subsequent studies also investigated GH release; however, the directionality of GH changes has not always been consistent, and the contribution of GH remains uncertain (7). Additional studies have suggested that reduced levels of insulin-like growth factor 1 (IGF1), the main effector of GH action on somatic growth, might be responsible, at least in part, for the growth defects in patients with CF (8, 9) and in CF mice (10).

Although much attention has been focused on growth deficits in children, adolescents, and adults, evidence suggests that individuals with CF are already different at birth. For example, several studies indicate that CF newborns weigh less, are shorter, and have a smaller head circumference than non-CF newborns do (2, 11, 12). The biological basis for these differences has not been identified.

We recently targeted the porcine *CFTR* gene and generated *CFTR*<sup>-/-</sup> and *CFTR*<sup>-/ $\Delta$ F508</sup> pigs (hereafter called “CF pigs”)

(13, 14). Newborn CF pigs display defective Cl<sup>-</sup> transport, meconium ileus, pancreatic destruction, and early focal biliary cirrhosis that replicate disease in newborn humans with CF (14). At birth, lungs of CF pigs lack inflammation, but, with time, they spontaneously develop infection, inflammation, and remodeling (15). This model provided us with an opportunity to ask whether CF pigs also manifest a growth defect. When we found that they did, we tested the hypothesis that CF pigs have reduced IGF1 levels.

## Results

**CF Pigs Show Reduced Growth and Lower IGF1 Levels.** All newborn CF pigs have meconium ileus (14). In humans, surgery is often required to treat the intestinal obstruction, and we therefore gave CF pigs (and littermate controls) an ostomy to circumvent the obstruction (15). We measured weight in five CF and seven non-CF pigs over time and found that CF pigs gained less weight than non-CF controls did (Fig. 1A).

Knowing the association between reduced IGF1 and growth in humans with CF, we measured serum IGF1 levels (Fig. 1B). Wild-type pigs showed the expected increase in IGF1 with time. In contrast, levels were lower in CF pigs.

**Newborn CF Pigs Have Reduced IGF1, Bone Length, and Bone Mineral Content.** Although malnutrition caused by pancreatic insufficiency and chronic pulmonary inflammation might limit growth later in life (16, 17), these factors will not have contributed at the time of birth because fetal nutrition is from the mother and chronic lung infection and inflammation begin after birth. Moreover, IGF1 does not cross the placenta (18). Therefore, to learn whether lack of cystic fibrosis transmembrane conductance regulator (CFTR) might have a more direct effect on growth, we measured serum IGF1 levels at the time of birth and found that they were reduced in CF pigs compared with littermate controls (Fig. 1C).

Reduced IGF1 levels were predictors that newborn CF pigs would show evidence of impaired in utero growth. Although, on average, CF piglets weighed 14% less than their non-CF littermates did, the difference was not statistically significant (Fig. 1D). Because IGF1 is important for bone size (19), we also measured humerus length. Newborn CF pigs had a humerus length that was ~9% shorter than that of non-CF controls (Fig. 1E). Likewise, bone mineral content and area were reduced in humeri from

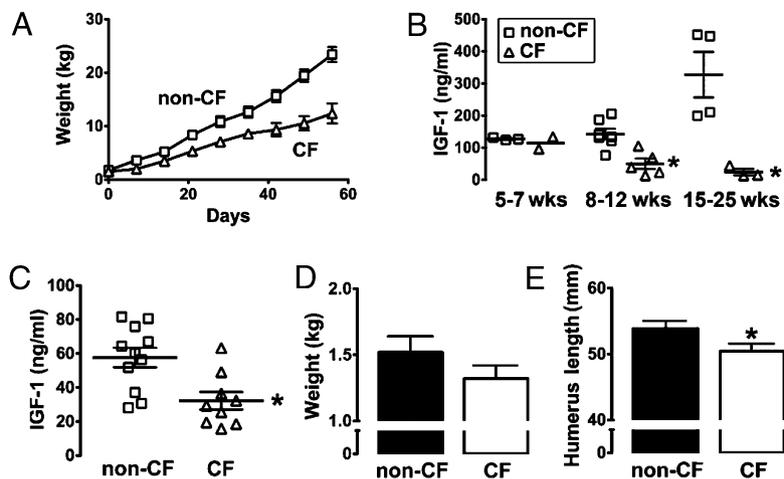
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Conflict of interest statement: Michael Welsh holds equity in Exemplar Genetics, which is licensing materials and technology related to this work.

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**Fig. 1.** IGF1 deficits and growth parameters in CF pigs. (A) CF pigs ( $n = 5$ ) gained less weight than did non-CF pigs ( $n = 7$ ). Data are mean  $\pm$  SE. (B) CF pigs ( $n = 5$ ) had reduced plasma IGF1 levels compared with non-CF pigs ( $n = 7$ ). Differences became more exaggerated over time. CF pigs, two females and three males; non-CF pigs, three females and four males. Data are for individual animals; line represents mean  $\pm$  SE. \* $P = 0.003$  compared with non-CF pigs at 8–12 wks and  $P = 0.016$  at 15–25 wks versus non-CF pigs. (C) Plasma IGF1 levels in newborn piglets 6–12 h old indicated that CF piglets (five females and four males) had significantly reduced plasma IGF levels relative to non-CF piglets (seven females and four males);  $P = 0.005$ . Data are for individual animals; line represents mean  $\pm$  SE. (D) Average birth weight was lower, but not statistically different, in CF compared with non-CF piglets ( $P = 0.12$ ). (E) Humerus length of newborn CF piglets was shorter than that of non-CF animals ( $P = 0.04$ ). For D and E, CF, 7 females and 11 males; non-CF, 7 females and 10 males. Data are mean  $\pm$  SE.

newborn CF pigs (Table 1). In contrast, bone density, which shows little relationship to cord blood IGF1 (20), did not differ by genotype. Decreased bone mineral density has been reported in CF mice, but these studies have tended to focus on older mice (21–24). Whether bone mineral density will decrease with time in CF pigs and whether IGF1 will contribute to this process remain to be determined. However, finding a growth defect in utero suggests a potential physiological consequence of decreased IGF1 in newborn CF piglets.

**Pituitary of CF Pigs Shows Reduced GH Secretion.** Circulating IGF1 is synthesized and released predominantly from hepatocytes; yet CFTR is primarily expressed in the apical membrane of bile duct cells (25). Thus, lack of CFTR in the liver seems unlikely to be the source of reduced circulating IGF1. Because IGF1 synthesis is under somatotrophic axis control, we tested for *CFTR* mRNA and found it in hypothalami and pituitaries of newborn pigs (Fig. 2A) (26). This result suggested that expression of key somatotrophic hormones might be altered; however, we detected no differences in mRNA for somatostatin or GH-releasing hormone in the hypothalamus, GH in the pituitary, or IGF1 in the liver (Fig. 2B–E). Because previous data suggested that CFTR might affect neuropeptide release (27), we reasoned that GH release might be impaired. To test this hypothesis, we cultured organotypic pituitary slices from CF and non-CF pigs and measured GH release after adding forskolin to elevate cellular cAMP levels (27). Forskolin induced a greater GH increase in non-CF than in CF pituitary slices (Fig. 2F and G). These data suggest that lack of CFTR

impairs release of GH, which may explain, in part, the reduced IGF1 levels.

**Human Newborns with CF Have Reduced IGF1 Levels.** Our discovery that IGF1 levels are reduced in CF pigs at birth suggested that the same might be true for humans. In Iowa, a blood spot is collected from newborns for genetic screening for CF and other inherited diseases. Previous work demonstrated the utility and validity of measuring IGF1 in dried blood spots (28). Therefore, we measured IGF1 levels in blood spots and found that newborns with CF had a small but significant reduction in IGF1 levels relative to non-CF babies (Fig. 3). These findings may explain the long-standing observation that CF babies are smaller than non-CF babies are at birth (2, 11, 12).

## Discussion

IGF1 is critical for both fetal and childhood growth (29). Serum IGF1 concentrations in the fetus correlate with fetal size (30), and alterations in the genes encoding IGF1 and the IGF1 receptor retard fetal growth (31–34). Targeted disruption of the mouse *IGF1* and *IGF* genes also markedly reduces fetal growth (35, 36). Although IGF1 levels are reduced in CF (8, 9), the temporal and/or causal relationships between lower IGF1 levels and growth defects and disease in CF have remained uncertain. Previous studies proposed that malnutrition and chronic pulmonary inflammation reduce IGF1 (37, 38). Our data do not argue against the contribution of these factors in older children and adults where IGF1 levels have previously been measured (8, 9, 39). However, our results in newborn pigs indicate that CFTR loss has an additional, more direct effect in reducing IGF1 levels. Moreover, in older patients and CF pigs, perhaps the combination of the effect we report plus the secondary effects on IGF1 levels related to disease progression might reduce IGF1 levels more than either alone.

Loss of CFTR function in the pituitary may contribute to reduced IGF1 levels in newborns (40). Consistent with this hypothesis, lack of CFTR expression decreased forskolin-stimulated release of GH from pituitary slice cultures. Although earlier studies postulated that older patients with CF have alterations in the somatotrophic axis, the location of the defect is uncertain (41), and there has been disagreement about the direction of changes

**Table 1. Humerus bone morphometry and density in newborn non-CF and CF pigs**

Parameter	Non-CF	CF	<i>P</i> value
Total content (mg/mm)	30.8 $\pm$ 3.6	20.8 $\pm$ 2.1	0.02
Total density (mg/cm <sup>3</sup> )	270.0 $\pm$ 7.1	266.9 $\pm$ 5.2	0.73
Total area (mm <sup>2</sup> )	110.3 $\pm$ 11.0	78.2 $\pm$ 8.4	0.03
Trabecular area (mm <sup>2</sup> )	89.5 $\pm$ 10.1	60.6 $\pm$ 7.6	0.03
Cortical/subcortical area (mm <sup>2</sup> )	20.9 $\pm$ 1.1	17.6 $\pm$ 0.8	0.03



infants, and, as discussed above, the consequences of IGF1 administration in this age group are not known. Perhaps newborn CF pigs might provide a model for testing this therapeutic approach. If additional larger studies reveal reduced IGF1 levels in newborn patients with CF and suggest a relationship with body size and disease severity, then it may be worth considering early replacement of IGF1 or GH for patients with CF, much as we replace their pancreatic enzymes and insulin.

## Materials and Methods

**Animals.** We previously reported generation of *CFTR*<sup>+/-</sup> and *CFTR*<sup>+/ $\Delta$ F508</sup> pigs, and production of *CFTR*<sup>-/-</sup> and *CFTR*<sup>-/ $\Delta$ F508</sup> pigs (13, 14). Animals were mated, and progeny was studied. Two different cohorts of animals were used for these studies. (i) Newborn piglets were used for studies of newborn IGF1 levels, birth weight, bone morphometric indices, and pituitary cultures. Only *CFTR*<sup>-/-</sup> pigs were used for studies at birth. Newborn piglets were separated from the sow within 6 h of birth. Piglets were weighed, sedated with ketamine and xylazine, and euthanized with an i.v. overdose of pentobarbital/phenytoin. (ii) Older pigs were used for studies of weight gain and IGF1 levels measured over time. Because of the limited number of animals surviving surgical correction of the meconium ileus, our studies of older pigs used both *CFTR*<sup>-/-</sup> ( $n = 3$ ) and *CFTR*<sup>-/ $\Delta$ F508</sup> ( $n = 2$ ) genotypes. Within 8–12 h after birth, control and CF piglets underwent placement of an ileostomy or cecostomy to prevent complications from meconium ileus. In the perioperative period, animals were maintained on i.v. fluids and prophylactic antibiotics. Thereafter, piglets were fed milk replacer and weaned to a solid diet. Animals received daily pancreatic enzymes, fat-soluble vitamins, an H2 blocker or a proton-pump inhibitor, and polyethylene glycol. Standard procedures for animal husbandry were used. All animal experiments were approved by the Institutional Animal Care and Use Committees of the Universities of Iowa and Missouri.

**IGF1 Measurements.** Serum was obtained from newborn piglets within 6–12 h of birth and periodically from older pigs and stored at  $-20^{\circ}\text{C}$  until analysis of duplicate samples. In the active IGF1 ELISA, IGF1 was separated from its binding protein in serum by using an ethanol and HCl-based extraction solution provided by the manufacturer (Diagnostic Systems Laboratories). Extraction proceeded for 30 min followed by a neutralization step. The neutralized sample was then spun briefly for 3 min at  $8,176 \times g$ , and the clear supernatant was used for the ELISA. The minimal detection limit was 0.03 ng/mL. The intra-assay and interassay coefficients of variation were 5.1% ( $n = 4$ ) and 5% ( $n = 4$ ), respectively.

IGF1 levels were measured in blood spots obtained from non-CF and CF infants born between 2007 and 2009. CF infants were detected by the Iowa Neonatal Metabolic Screening Program. All studies were approved by the University of Iowa Institutional Review Board and the Congenital and Inherited Disorders Advisory Committee of the Iowa Department of Public Health. A diagnosis of CF was confirmed by presence of two *CFTR* disease-causing mutations, elevated sweat chloride levels, and/or abnormal nasal voltage measurements. Of 23 CF infants, 8 were *CFTR* $\Delta$ F508/ $\Delta$ F508. Of the remaining 15 CF infants, 11 had one *CFTR* $\Delta$ F508 allele, and 5 had at least one *CFTR*-R117H mutation. Control blood spots were randomly obtained from samples collected over the same time period, but they had normal immunoreactive trypsinogen levels. All blood spots were frozen from time of collection. The dried blood spot samples (8-mm punch) were extracted and analyzed for IGF1 by using Diagnostic Systems Laboratories ACTIVE non-extraction IGF-I ELISA. All measurements were performed by ZRT Laboratory.

**Bone Morphometry Measurements.** Pig forelimbs were removed from 18 CF and 17 non-CF pigs and stored in 10% formaldehyde. The humeri were dissected and measured lengthwise from the humeral head to the groove between the medial and lateral epicondyle with digital calipers (Fisher Scientific).

**Peripheral Quantitative Computed Tomography.** Humeri were placed in an XCT 3000 Research (STRATEC Biomedical Systems) scanner, and an axial scout scan was performed. A reference line was established at the distal end of the humerus, and a 2-mm-thick slice was positioned 9 mm from the proximal end

of the bone (metaphyseal region). Bone mineral content, density, and area were calculated by using standard CalcBD and CortBD routines. Tissue measuring  $\leq 200 \text{ mg/cm}^3$  was classified as soft tissue. Trabecular (cancellous) bone threshold was between 200 and  $500 \text{ mg/cm}^3$ . Cortical (compact) bone was represented by values higher than  $700 \text{ mg/cm}^3$ .

**RT-PCR.** The liver, pituitary, and brain were rapidly removed and placed into RNA later solution (Ambion). Two horizontal cuts on the ventral surface of the brain (one just caudal to the optic chiasm and another made rostral to the mammillary bodies) were made, and the hypothalamus was removed. Total RNA was isolated with the Qiagen Lipid Tissue RNeasy kit. RNA quality and concentration were assessed via Nanodrop. RNA was reverse-transcribed with the RT<sup>2</sup> First Strand Kit (SA Biosciences). Quantitative RT-PCR was used to measure mRNA. The following primer sequences were used: somatostatin (forward, 5'-ATCGTCCTGGCTTTGGGC-3'; reverse, 5'-GCCTCATCTCGTCCTGCTCA-3') (59); GH-releasing hormone (forward, 5'-TATGCAATGCCATCTTC-3'; reverse, 5'-AAGCCGTACCTTGCTCC-3') (60); GH (forward, 5'-GTTTGGCACCTCAGACCG-3'; reverse, 5'-CCAGCACTAGAAAGGCACAGC-3') (60); IGF1 (forward, 5'-CACATCACATCCTTCG-3'; reverse, 5'-CTGGAGCCGTACCTGTG-3'); and actin (forward, 5'-CTGCGGCATCCAGAACT-3'; reverse, 5'-GTGATCTCTTCTGCATCCTGTG-3'). Primer efficiency and validation were performed as described in ref. 61. PCR was performed in sample triplicates with RT<sup>2</sup> SYBR Green qPCR Master Mixes (SA Biosciences) and a 7500 Fast Real-Time PCR System (Applied Biosystems), following manufacturers' protocol. RNA not reverse-transcribed was used as control. Standard delta delta CT method with actin as a reference was used for analysis. For porcine *CFTR*, amplification consisted of 35 cycles at  $94^{\circ}\text{C}$  for 5 s,  $55^{\circ}\text{C}$  for 5 s, and  $72^{\circ}\text{C}$  for 15 s, and a final extension cycle of  $72^{\circ}\text{C}$  for 5 min with the following primers: forward, 5'-CTGGAGCCTCAGAGGGTAAAT-3'; reverse, 5'-AGTTGGCAGCCTTGATGACTCC-3'. Products were separated on a 1.7% agarose gel.

**Organotypic Pituitary Slice Culture and GH Measurement.** Whole pituitaries of newborn CF and non-CF pigs were quickly removed and dissected into 350- $\mu\text{m}$ -thick coronal sections by using a manual tissue chopper. Slices were placed in polyethylene terephthalate-etched membrane culture inserts containing 1- $\mu\text{m}$  pores (Fisher Scientific). Slices were cultured in 25% horse serum, 25% HBSS, 50% MEM, 2 mM glutamine, 1 mg/mL glucose, 44 mg/mL  $\text{NaHCO}_3$ , and 10 units/mL penicillin-streptomycin in a 5%  $\text{CO}_2$  humidified incubator. On day 6 or 7, medium was changed and slices were incubated in serum-free medium (MEM replaced serum, and osmolarity was adjusted with bovine albumin fraction V to 1.5%) for 3 h. A 100- $\mu\text{L}$  sample of culture medium was collected to measure basal GH levels, followed by addition of forskolin (0.5  $\mu\text{M}$ ) to stimulate release of GH (62). At 30 min later, another 100- $\mu\text{L}$  sample of culture medium was collected. GH levels were measured by ELISA (Millipore) in duplicate samples. Porcine GH (obtained from A. F. Parlow of the National Institute of Diabetes and Digestive and Kidney Diseases' National Hormone and Peptide Program) was used to construct standard curves. The minimal detection limit was 0.07 ng/mL. The intra-assay and interassay coefficients of variation were 3.8% ( $n = 4$ ) and 4% ( $n = 4$ ), respectively.

**Statistical Analysis.** Results are expressed as mean  $\pm$  SE. For IGF1 analysis in older pigs, a one-way ANOVA was performed, followed by a Fisher's least significant difference post hoc test to determine the source of significance. For all other measurements, statistical differences between CF and non-CF were evaluated with a two-tailed unpaired  $t$  test. Statistical significance was determined by  $P \leq 0.05$ . A Grubbs test was performed once to detect statistical outliers.

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