Human stanniocalcin-2 exhibits potent growth-suppressive properties in transgenic mice independently of growth hormone and IGFs

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Submitted 22 June 2004; accepted in final form 9 September 2004

Human stanniocalcin-2 exhibits potent growth-suppressive properties in transgenic mice independently of growth hormone and IGFs. Am J Physiol Endocrinol Metab 288: E92–E105, 2005. First published September 14, 2004; doi:10.1152/ajpendo.00268.2004.—Stanniocalcin (STC)-2 was discovered by its primary amino acid sequence identity to the hormone STC-1. The function of STC-2 has not been examined; thus we generated two lines of transgenic mice overexpressing human (h)STC-2 to gain insight into its potential functions through identification of overt phenotypes. Analysis of mouse Stc2 gene expression indicates that, unlike Stc1, it is not highly expressed during development but exhibits overlapping expression with Stc1 in adult mice, with heart and skeletal muscle exhibiting highest steady-state levels of Stc2 mRNA. Constitutive overexpression of hSTC-2 resulted in pre- and postnatal growth restriction as early as embryonic day 12.5, progressing such that mature hSTC-2-transgenic mice are –45% smaller than wild-type littersmates. hSTC-2 overexpression is sometimes lethal; we observed 26–34% neonatal morbidity without obvious dysmorphology. hSTC-2-induced growth retardation is associated with developmental delay, most notably cranial suture formation. Organ allometry studies show that hSTC-2-induced dwarfism is associated with testicular organogemat and a significant reduction in skeletal muscle mass likely contributing to the dwarf phenotype. hSTC-2-transgenic mice are also hyperphagic, but this does not result in obesity. Serum Ca2+ and PO4 were unchanged in hSTC-2-transgenic mice, although STC-1 can regulate intra- and extracellular Ca2+ in mammals. Interestingly, severe growth retardation induced by hSTC-2 is not associated with a decrease in GH or IGF expression. Consequently, similar to STC-1, STC-2 can act as a potent growth inhibitor and reduce intramembranous and endochondral bone development and skeletal muscle growth, implying that these tissues are specific physiological targets of stanniocalcins."
lie primarily on intracellular pools rather than systemic Ca²⁺ regulation.

We and others have attempted to gain further insight into the function(s) of STC-1 through the generation of gain-of-function transgenic mice (13, 49). It was anticipated that overexpression of STC-1 would result in a deleterious phenotype(s) that would point to specific organ systems particularly sensitive to STC-1 signaling and therein provide the basis for further study of STC-1 action in a specific cell type. This approach was predicated on the fact that STC-1 is not detectable in serum, other than during pregnancy in the mouse (11). Our studies (49) indicated that ubiquitous overexpression of hSTC-1 results in permanent and severe postnatal dwarfism, with transgenic mice achieving body weights 30–45% less than their wild-type counterparts. Using a similar hSTC-1-transgenic mouse model, others (13) observed a less severe dwarf phenotype along with decreased bone formation and somewhat altered bone structure. Congruent with a role for STC-1 in bone formation, a recent study (59) showed that it can stimulate the differentiation of rat calvaria cell cultures, implying that STC-1 is an autocrine/paracrine modulator of osteoblast development. These studies strongly suggest that STC-1 signaling can affect multiple organ systems and that it is a regulator of organ development with potent growth inhibitory properties when present in excess.

Because it is increasingly clear that STC-1 plays important physiological roles in mammals, we postulate that a related protein, STC-2, has an equally important function(s) in mammals. Given the distinct biochemical and gene expression pattern differences between STC-1 and STC-2, we chose mouse transgenesis to determine whether STC-2 could regulate physiological pathways separate from those controlled by STC-1 and thus produce unique phenotypes. Our data indicate that STC-2 mRNA levels are markedly lower than those of STC-1 during mouse development and that it is most highly expressed in heart followed by skeletal muscle, uterus, and prostate. We have generated transgenic mice that ectopically produce hSTC-2 early in development, when it is not normally expressed, and throughout adulthood. Here, we describe that STC-2 can cause early intrauterine growth restriction, developmental delay, and severe postnatal growth retardation with disproportional organ growth. This growth inhibition appears to be independent of the growth hormone and IGF-I and -II growth regulatory pathway. These results are the first to show that STC-2 can, in an overexpression model, induce physiological effects similar to STC-1, resulting in similar but more severe phenotypes.

MATERIALS AND METHODS

Generation of hSTC-2-transgenic mice. The hSTC-2 transgene was constructed by ligation of a 1,247-bp hSTC-2 cDNA fragment from expressed sequence tag (EST) H98185 (Genome Systems, St. Louis, MO) encompassing the 909-bp coding sequence into the XhoI site of the previously described pCAGGS expression vector (34). Expression of the transgene is controlled by the bipartite promoter, consisting of the 384-bp cytomegalovirus immediate early (IE) enhancer fused to the 284-bp chicken β-actin promoter contiguous with 83 bp of the chicken β-actin 5′-untranslated sequence (UTR), followed by the 917-bp chicken β-actin intron 1. The XhoI cloning site is located in the rabbit β-globin 3′-UTR of the expression cassette. The expected size of the transgene-derived hSTC-2 transcript is ~1.8 kb, and it is easily distinguishable by Northern analysis from the ~4-kb primary endogenous mSTC-2 mRNA. The pCAGGS/hSTC-2-transgenic mice were generated in the Transgenic and Gene Targeting Facility of the London Regional Cancer Centre by microinjection of purified transgene DNA into the pronuclei of fertilized C57BL/6 × CBA oocytes, as previously described (4). Transgenic founder mice were identified and genotyped by dot-blot hybridization with genomic DNA from tail biopsies and a radiolabeled 634-bp PstI/Styl hSTC-2 cDNA fragment encompassing 617 bp of the coding sequence and 17 bp of 3′-UTR (49). Southern blot analysis was performed with hSTC-2-transgenic mouse genomic DNA isolated from adult kidney to confirm intact integration of the transgene. hSTC-2- and hSTC-1-transgenic mouse lines were maintained on a C57BL/6 × CBA background. All studies were performed with mice hemizygous for the pCAGGS/hSTC-2 transgene. Mice were housed and used in accordance with protocols approved by the University Council on Animal Care at the University of Western Ontario.

Serum hSTC-2 determination and blood chemistry. Antibodies to hSTC-2 were prepared in rabbits after three monthly immunizations, each of 20 µg of Chinese hamster ovary cell-expressed recombinant hSTC-2 dissolved in Freund’s adjuvant in saline (1:1, vol/vol). Whole blood was taken from transgenic and wild-type mice immediately after CO₂ asphyxiation by opening the abdomen and collecting blood, using a 25-gauge needle, from the caudal vena cava. Blood was allowed to coagulate at room temperature, and serum was collected as the supernatant from two consecutive 18°C 20-min centrifugations at 15,000 × g. Mouse serum hSTC-2 was characterized by separating 3 µl of a 1:10 dilution or 1 µl of serum proteins by SDS-PAGE in 12% gels. These gels were Western blotted with the use of a 1:5,000 dilution of rabbit anti-hSTC-2 antisera raised by Veterinary Services, University of Western Ontario, against recombinant hSTC-2 provided by Human Genome Sciences (Rockville, MD) (60). The apparent molecular weight of transgene-derived hSTC-2 was compared with hSTC-2 produced by the MCF-7 human breast carcinoma cell line by Western blotting. MCF-7 cells were cultured under serum-free conditions for 3 days, and the conditioned medium was collected and concentrated sixfold with Centricron YM-10 centrifugal filter devices (Millipore, Billerica, MA). Mouse serum IGF-I was measured with a rat IGF-I RIA ( Diagnostic Systems Laboratories, Webster, TX) after acid-ethanol extraction of 50-µl serum samples from 6-wk-old wild-type and hSTC-2-transgenic mice. Serum IGF-I measurements are presented as means ± SE. Blood chemistry determinations (Ca²⁺, PO₄, alkaline phosphatase) were carried out with the Synchron Clinical System CX7 and LX20 (Beckman Coulter, Brea, CA) at the London Health Sciences Center (London, Canada).

Northern blot analysis. Mice were CO₂ asphyxiated, and tissues were removed and extracted in TRIzol (Life Technologies, Grand Island, NY) for the isolation of total RNA. All RNA samples were subjected to Northern blot analysis using the 32P random primer-labeled 634-bp hSTC-2 cDNA fragment described above (50). Total RNA was pooled from three to five animals or embryos for transgenic or endogenous mSTC-2 expression studies. Total RNA, isolated from 5–10 pooled male pituitaries, was blotted and hybridized with a mouse growth hormone (GH) cDNA fragment. IGF-II mRNA levels were assessed by blotting pooled total RNA from three to five embryos at embryonic day (E)14.5, E16.5, and E18.5 and hybridization with a mouse IGF-II cDNA fragment. Pooled liver total RNA from three to five mice aged 5, 10, and 15 days was blotted and hybridized with a rat IGF-I cDNA fragment to determine postnatal IGF-I mRNA levels. To normalize for RNA loading and to determine fold changes in steady-state mRNA levels, blots were hybridized to 18S ribosomal DNA, and the signal was quantified using PhosphorImager and ImageQuant software (Amersham Biosciences, Piscataway, NJ).

Major urinary protein analysis. Urine was collected from age-matched hSTC-2-transgenic and wild-type mice, and 2 µl of urine were analyzed by SDS-PAGE in 12% gels, which were then stained with Coomassie blue.
Analysis of hSTC-2-transgenic and wild-type mouse weight gain.

Growth studies were conducted on each hSTC-2-transgenic line with wild-type littermates as controls. Hemizygous transgenic male mice were bred with wild-type females to generate timed-pregnant females carrying mixed litters of wild-type and transgenic pups. Embryos were harvested at E12.5, E14.5, E16.5, and E18.5 and dissected from the conceptus, and amniotic fluid was removed by blotting on paper towels. Wet weights were recorded, and the embryos were genotyped as described above using a portion of the embryo for DNA isolation. Human STC-1-transgenic embryo weights were determined similarly, except that wild-type females were bred to homozygous hSTC-1-transgenic males from lines 2 and IA to generate litters of hemizygous transgenic embryos, and wild-type embryo weights were obtained from wild-type crosses. For postnatal growth studies, pups were numbered at birth with a surgical marker and weighed between 8:00 and 10:00 AM on postnatal day (P)1–P17 and P20, P21, P25, P30, and P45. Toes were clipped on P10, and numbered ear tags were applied on P21 to track the mice. Mice were weaned at 21 days of age and transferred to separate wild-type and transgenic mouse stock cages for the remainder of the study period. For line L314 (L314), 25 transgenic females and 30 transgenic males were followed, and for line L372 (L372), 40 transgenic females and 39 transgenic males were included in the growth study. Seventy wild-type males and an equivalent number of wild-type females were used in this study. The growth study was extended to 80 days for L314 hSTC-2-transgenic mice only.

Neonatal morbidity. New litters were carefully monitored from four different matings: hemizygous hSTC-2 matings were established within each transgenic line to produce pregnant transgenic females; wild-type C57BL/6 females were housed individually and monitored as described above. When litters were discovered, wild-type pups (~1.4 g) were distinguished from transgenic pups (~1 g) by their weight and eliminated from the litter. The average transgenic litter size was 3.8 for each line, and 25 litters were followed from L372 and 27 from L314. Litters were monitored daily, and dead pups were retrieved for genotyping to confirm transgenic status.

Organ allometry. Fourteen-week-old wild-type and L314 hSTC-2-transgenic male mice were CO2 asphyxiated and weighed, and external body dimensions (nose-to-tail tip, nose-to-anus, and anus-to-tail lengths) were determined, followed by the removal of the internal organs for wet weight measurements. Organs were weighed on a Mettler PB303 DeltaRange balance immediately upon dissection, with the exception of the heart and lungs, which were briefly blotted on paper towel to remove excess blood. The remaining viscera were removed, briefly blotted dry, and weighed, and then the remaining carcass was weighed. Weighing both the anterior and posterior muscle groups of the hindlimbs and combining the weights of the left and right leg muscles determined the muscle weights of 12-wk-old animals. Tail weight for each animal was also determined after dissection from the body at the base of the tail. Organ and muscle weights were normalized to the intact body weight of the mouse and expressed as a relative percentage of body weight. This was done because a comparison of raw measurements between wild-type and transgenic mice was not feasible, given that the transgenic animals were dwarves. For the weight-matched wild-type mouse organ analysis, 26-day-old wild-type males were used because their body weight was closely matched that of 14-wk-old hSTC-2-transgenic males. Organs were dissected and weighed as described above. The dry weight of testes harvested from hSTC-2-transgenic and age-matched wild-type animals was obtained by placing pairs of testes from each animal in glass drying dishes and first obtaining wet weight. Testes were then incubated at 65°C until weights were constant (3 days), indicating no further detectable loss of water.

To obtain an indication of the relative mass of the skeleton, 14-wk-old hSTC-2-transgenic males and females from L314 and age-matched wild-type animals were subjected to dual-energy X-ray absorptiometry (DEXA) using a PIXI-mus Small Animal Densitometer (Lunar, Madison, WI). This analysis was performed at the Centre for Modeling Human Disease Physiology Core at the Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Canada. The procedure includes the mouse tail and head in the image and analysis. DEXA bone mineral content measurements show high correlation to the total ashed weight of bone (r = 0.99) (5, 33); therefore, when normalized to body weight, DEXA provided a measurement of ashed skeletal mass derived from mineral content. The percent fat mass and lean mass of each animal were also determined using DEXA.

Analysis of mouse embryo fibroblast proliferation. To establish an in vitro model of the intrauterine growth retardation phenotype observed for transgenic embryos, we prepared mouse embryonic fibroblasts (MEFs) from E14.5 hSTC hemizygous transgenic litters and C57BL/6 × CBA wild-type littermates (16). Cell number was obtained using an electronic particle counter (Beckman Coulter, Hialeah, FL), and MEFs were seeded at a density of ~5 × 10^4 cells/ml and allowed to attach to 175-cm^2 culture flasks in triplicate. The zero time point cell number was obtained 5 h after the initial plating to take into consideration potential plating efficiency differences and obtain starting cell numbers based on adherent cells rather than the cell seeding number. Day 0 mean cell numbers were 61,760 and 70,380 for wild-type and transgenic cultures, respectively. Thereafter, the cell number was determined every 24 h over the 11-day assay. For the high-density proliferation study, MEFs were plated at 1 × 10^5 cells/10-cm dish in triplicate and assayed as described above. MEF proliferation characteristics over the study period were determined by calculating the fold change in cell number per day by dividing the total cell number on a specific day by the total cell number on the previous day. All experiments were performed with three to five different preparations of MEFs.

Assessment of cranial suture development. Skeletal preparations of newborn P1 pups were prepared as previously described (28) after CO2 asphyxiation. Once the skeletons had been stained with Alcian blue and Alizarin red, the heads were removed below the base of the skull. The anterior surface of the skulls was photographed with an Olympus microscope (Olympus America, Melville, NY) equipped with a digital camera (Dage-MTI, Michigan City, IN) and Image-Pro Plus 4.5.1 computer software (MediaCybernetics, Silver Spring, MD). The magnification of all skull images was ×10, The area of cranial patency, or the open area between the cranial suture edges, was determined as pixel area by tracing along the edge of the sutures enclosing the space between the skull plates using OpenLab 3.1 software (Improvision, Lexington, MA).

Food intake. To compare food consumption in hSTC-2-transgenic and wild-type mice, we used 14-wk-old L314 transgenic males, 14-wk-old wild-type males, and 27-day-old wild-type males for the weight-matched comparison. Mice were housed in individual shoebox cages equipped with a pellet-feeding tube attached with a tube clip (Bio-Serv, Frenchtown, NJ). The mice were allowed to acclimate in the cage for 2 days with ad libitum access to regular mouse chow and water. On day 0 of the study, grain-based pellets (Bio-Serv) were loaded into the feeding tube, and the tube/food apparatus was weighed. Each morning for 7 days, the tube containing the food pellets was weighed to calculate the difference from the previous day.
RESULTS

Expression pattern of STC-2 in mature wild-type mice. Our analysis of mouse embryos indicated that, unlike STC-1, STC-2 mRNA was not detectable in whole embryo RNA from E10.5 to E18.5 (data not shown). In adult mouse tissues, Stc2 gene expression was detectable as two distinct mRNA species of ~4 and 2 kb (Fig. 1). STC-2 mRNA appeared most abundant in heart, prostate, uterus, and skeletal muscle. Lower, but detectable, STC-2 mRNA was present in seminal vesicle, ovary, mammary gland, and white fat depots. Generally, our observations of STC-2 gene expression indicate overlap with STC-1 expression, but the level of expression for each hormone was distinct and tissue dependent. For example, we could not detect STC-2 mRNA in the mouse ovary, whereas STC-1 mRNA is readily detectable by Northern blot (data not shown).

Generation of transgenic hSTC-2 mice. hSTC-2 overexpression was achieved by use of the bipartite cytomegalovirus (CMV) IE enhancer fused to the chicken β-actin promoter (Fig. 2). Southern blotting to confirm intact integration of the transgene revealed that two independent lines of transgenic mice were successfully created, L314 and L372 (data not shown). Both founders stably transmitted the transgene to progeny, thereby establishing two independent lines of hSTC-2-transgenic mice.

hSTC-2 transgene expression is widespread. Our first objective was to determine the pattern of hSTC-2 transgene expression and whether hSTC-2 was detectable in the serum of transgenic mice. Transgene expression was detectable by Northern blotting of whole embryo RNAs from E10.5 onward in both lines of mice, whereas no signal was observed in the wild-type embryo (Fig. 3A). Northern blotting of adult transgenic tissue RNAs from both lines indicated that all tissues tested contained the expected ~2-kb mRNA corresponding to the transgene-derived hSTC-2 transcript (Fig. 3, B–D). The highest levels of transgene expression were seen in the heart and skeletal muscles of both lines of hSTC-2-transgenic mice. Moreover, an analysis of transgene expression between the sexes in either line did not reveal a sex-specific difference (Fig. 3, B and C), as seen previously for hSTC-1-transgenic mice (49).

The antisera raised to hSTC-2 was characterized by Western blotting, using conditioned medium from human cell lines (Fig. 4A). The predicted and observed molecular mass of the mature secreted form of hSTC-2 is ~30.6 kDa (32). A major band of ~33 kDa and two larger minor bands were seen in concentrated conditioned media from the human MCF-7 breast carcinoma cell line and not from HeLa cells, which corroborates our Northern analysis of hSTC-2 gene expression in these cell lines. The antisera detected hSTC-2 in conditioned media from the rat GC pituitary tumor cell line, indicative of its ability to cross-react with rodent STC-2 (Fig. 4A), but did not detect STC-2 in wild-type mouse serum as we have reported previously for STC-1 (11). However, in transgenic mouse serum, the antisera revealed 31- and 35-kDa hSTC-2 bands, possibly due to different posttranslational modifications (Fig. 4B). By Western blot analysis, L314 serum contained approximately fivefold more circulating hSTC-2 than that of L372 (Fig. 4B).

hSTC-2-transgenic neonate viability is significantly reduced. While breeding the hSTC-2-transgenic mouse lines, it was clear that a large number of transgenic pups were lost soon after birth. Hemizygous transgenic males were bred with wild-type and hemizygous transgenic females to determine whether neonatal mortality was associated with transgene homozygosity in both lines. Regardless of the mating genotypes, neonatal morbidity ranged from 26 to 33.8% within the first few days of life (Table 1), indicating that neonatal death was not dependent on transgene homozygosity. Importantly, this was the first indication that excess production of a stanniocalcin could cause death. Moreover, the death of transgenic neonates was not due to an inability to compete with wild-type pups for breast milk, as the removal of wild-type pups from mixed litters did not alter neonatal morbidity (31.3% for L314 and 28.4% for L372).

Overexpression of stanniocalcins causes intrauterine growth restriction and postnatal growth retardation. This overt phenotype was first noticed in P1 pups; therefore, we examined embryo weights from E12.5 to E18.5 to determine whether hSTC-2 caused intrauterine growth restriction. The weights of hSTC-2-transgenic embryos were significantly less than wild-type embryos from E12.5 onward (Table 2). The difference in embryo size can easily be seen in the images of representative embryos and is evident as early as E10.5 (Fig. 5).

In a previous study with hSTC-1 transgenic-overexpressing mice (49), we also observed postnatal growth restriction. Therefore, in this study, we reassessed the growth of transgenic embryos from hSTC-1-transgenic lines 1A and 2 for intrauterine growth restriction. The lower-expressing line 1A, significantly smaller embryos were first detected at E14.5, and at E15.5 for line 2. Therefore, both stanniocalcins had similar growth inhibitory effects on embryonic growth that correlated with the onset of transgene activation.

To assess hSTC-2-induced postnatal growth restriction, we measured the body weights of wild-type and transgenic ani-
mals from both lines for a total of 45 days. Statistical analyses of the weight determinations indicated that the transgenic offspring remained significantly smaller than their wild-type littersmates throughout the study (Fig. 6A). By the end of the study, the L314 and L372 transgenic females were 43.5 and 41% smaller, respectively, than their wild-type female siblings. Similarly, the transgenic males were 40.3% (L314) and 45.2% (L372) smaller than their wild-type male littersmates. The growth study was extended to 80 days for L314, during which time the weight differential with wild-type littersmates was maintained (P < 0.0001) such that transgenic males and females were 46.8 and 43.2% smaller, respectively (data not shown).

These weight measurements were then used to assess weight gain per day to determine whether the pattern of growth of hSTC-2 transgenics was distinctly different from that of wild-type littersmates (Fig. 6B). Statistical analysis of the data revealed that the amount of weight gained per day by hSTC-2-transgenic mice was significantly (P = 0.003) less than their wild-type littersmates from P2 to P15. From P17 to P25, hSTC-2-transgenic pups experienced a slowdown in growth relative to their wild-type counterparts, and this corresponded to the adjustment period associated with weaning at P21. From P17 to P45, the gap between wild-type and transgenic mice in the amount of weight gained per day increased with age and remained statistically significant (P < 0.0001).

Plotting the body mass data as a growth rate curve was done to determine whether the hSTC-2-transgenic mice exhibited significantly slower growth rates compared with their wild-type littersmates (Fig. 6C). The transgenic mice generally exhibited a significantly slower rate of growth from P1 to P30; however, from P30 to P45, both sexes from each transgenic line displayed a higher growth rate than wild-type mice. Then, from P45 to P52, the growth rates of both transgenic and wild-type mice decreased to a constant rate. Interestingly, the growth rates of the wild-type and hSTC-2-transgenic mice were not significantly different from P45 to P73 but became different from P73 to P80, likely because wild-type mice gained additional weight due to fat deposition.

hSTC-2 overexpression can affect organ size. To determine whether hSTC-2 affected the growth and morphology of specific tissues, we performed organ allometry studies on 14-wk-old hSTC-2 L314 males and normalized the wet organ weights to body weight for comparison with sex- and age-matched wild-type littersmates. The hSTC-2-transgenic males were 45% smaller than their wild-type littersmates. hSTC-2-transgenic males were significantly smaller than their age-matched wild-type counterparts in total length (3 cm), body length (1.6 cm), and tail length (1.7 cm) (Table 3). Statistical analyses of the normalized data showed that the brain, kidney, liver, heart, lung, and viscera of hSTC-2-transgenic males comprised a larger percentage of body weight compared with age-matched wild-type males (Table 4). The largest difference in normalized organ weights was seen for hSTC-2-transgenic testes that were 72% larger than expected and essentially identical in weight to those from wild-type male mice. Fluid accumulation in the transgenic testes did not account for the greater-than-expected weight, as the dry weights of transgenic and wild-type testes were 0.022 and 0.024 g, respectively (P = 0.2961).

After removal of the major organs and viscera, the remaining carcass consisting of skin, muscle, and skeleton was weighed, and the normalized data indicated that the hSTC-2-transgenic carcass was 8% smaller than expected (P < 0.0001). This small but statistically significant difference implied that the skeleton and/or skeletal muscle was negatively affected by hSTC-2 overexpression. To analyze the bone, we performed an age-matched comparison of whole skeletons from wild-type and hSTC-2-transgenic mice with DEXA. The data, when normalized to the whole body mass (43), revealed that there were no differences between wild-type and transgenic mice of both sexes, indicating that the change in carcass mass was not due to a disproportionally smaller skeleton (data not shown).

However, skeletal muscle clearly showed a 15–32% reduction (P < 0.05–0.0001), depending on the muscle group (Table 4). This implied that the growth-restrictive effect of hSTC-2 on the whole animal was in part due to a reduction in normal muscle growth.

Organ allometry was also performed with weight-matched wild-type mice to determine whether hSTC-2-transgenic mouse body composition was equivalent to a similar-size wild-type mouse. Because of the large difference in weight between 14-wk-old wild-type and hSTC-2-transgenic mice, this study necessitated the use of 26-day-old wild-type males to provide appropriately weight-matched mice (Table 4). In terms of linear measurements, the 14-wk-old hSTCtP-transgenic males were slightly bigger (0.53 cm) than the weight-matched wild-types because of a difference in tail length (Table 3). It is notable that, despite the longer tail length, hSTC-2-transgenic tail mass was 45% less than in weight-matched wild-type males (Table 3). We found that only the lung and heart absolute weights were not statistically different between transgenic and wild-type mice. Collectively, these data suggest that hSTC-2 expression results in a growth restriction phenotype.
where a 14-wk-old transgenic male typifies a 26-day-old wild-type mouse in terms of body proportions and organ mass.

Food intake by hSTC-2-transgenic mice exceeds that of wild-type mice. It is possible that the dwarf phenotype induced by hSTC-2 expression could in part be due to a significant change in food intake. On account of their age (14 wk), the average weight of age-matched wild-type and hSTC-2-transgenic mice did not change significantly over the 7-day study period, whereas weights of the younger weight-matched wild-type mice increased by 5 g. When food consumption was

Fig. 3. hSTC-2 transgene expression. A: transgene-derived hSTC-2 transgene mRNA was detectable by Northern blotting with 50 μg of whole embryo RNA, starting at embryonic day (E)10.5 in both lines. B–D: transgene hSTC-2 mRNA was detected in all tissues tested, with the highest levels found in the heart and skeletal muscle (B, C). L314 mice (B) exhibited higher levels of transgene expression than L372 mice (C), with no apparent difference in tissue expression pattern or between the sexes after 18S rRNA normalization (not shown). Total RNA (30 μg) pooled from 3 to 5 mice was analyzed, and the autoradiograms are from 24-h exposures. M, male; F, female; wt, wild type; Sem Ves, seminal vesicle.
examined as a function of body weight, it was clear that hSTC-2-transgenic males ate 22% more food than their age-matched wild-type counterparts (P < 0.05; Fig. 7). This result eliminates the possibility that higher-than-normal STC-2 production led to a behavioral defect that altered food consumption and ultimately caused undernourishment and delayed growth. DEXA analysis also revealed that female and male transgenic mice on average carried 1.8 and 2.4% more fat, respectively (Table 5). The higher fat content was not statistically significant compared with wild-type counterparts (Table 5). The higher fat content was not statistically significant compared with wild-type and hSTC-1-transgenic mice from both lines showed that L314 contained ~5-fold higher levels of hormone compared with L372. Tg, transgenic; TgF, transgenic female; TgM, transgenic male.

Table 1. Neonatal morbidity

| Mating       | No. of Pups | No. of Neonate Deaths | No. of Litters |
|--------------|-------------|-----------------------|---------------|
|              | Wild type   | Tg                    | Wild type     | Tg       |
| L314 het × het | 73 (29.2%) | 177 (70.8%)           | 6 (14.6%)     | 60 (33.8%) |
| L372 het × het | 40 (22.6%) | 112 (77.4%)           | 5 (12.5%)     | 30 (26.8%) |
| L314 TgF × wtM | 91 (56%)   | 65 (44%)              | 3 (3.2%)      | 17 (26%)  |
| L314 TgM × wtF | 154 (57.6%) | 113 (42.3%)          | 2 (1.5%)      | 28 (24.4%) |

Values in parentheses represent the resultant percentage of mice of the indicated genotype for each mating and the percentage of neonate deaths of that genotype. Tg, transgenic; wt, wild type; F, female; M, male; het, hemizygous.

Intramembranous and endochondral bone formation. To assess whether the dwarfism exhibited by the overexpression of hSTC-2 manifested itself at the level of gross skeletal development, the cartilage and bones of newborn (P1) mice were stained with Alizarin red and Alcian blue. On visual inspection it was strikingly apparent that the intramembranous bones of hSTC-2-transgenic neonates were less developed than the wild types (Fig. 8A). Transgenic mice exhibited severe cranial patency between the leading edge of the frontal and parietal bones compared with wild-type mice. Cranial patency area in hSTC-2-transgenic skulls was found to be 26,010 pixels compared with 12,620 pixels for wild-type skulls (P < 0.0001) (Fig. 8B). hSTC-1-overexpressing lines were also analyzed in this manner and found to exhibit a greater degree of cranial patency compared with wild-type mice (data not shown). With regard to endochondral bone formation, developmental delay was observed at E16.5 (Fig. 8C). The ilium, ischium, and pubic bones of the hip were ossified in the wild-type embryo, whereas little or no ossification is apparent in the hSTC-2-transgenic littermates (Fig 8C). Ossification is also lacking in the sacral vertebrae of the transgenic embryos.

hSTC-2 overexpression does not significantly affect serum Ca2+ and PO4. A number of reports have described effects of STC-1 on mineral metabolism (26, 37, 52). Therefore, we sought to determine whether excess hSTC-2 significantly altered serum levels of Ca2+ and PO4. To this end, 9-wk-old hSTC-2-transgenic and wild-type sera were analyzed, and only L314 transgenic females showed a significant decrease in serum Ca2+ (Table 6). However, serum alkaline phosphatase levels were significantly lower in hSTC-2-transgenic males and females, but only for the higher expressing L314 mice.

GH and IGF production in hSTC-2-transgenic mice is not altered. The obvious growth restriction induced by hSTC-2 overexpression suggested that STC-2 may interfere with expression of the primary growth-promoting trophic factors, GH, IGF-I, and IGF-II, thus accounting for the dwarf phenotype. To examine this possibility, we performed Northern analysis of steady-state GH mRNA levels in 6-wk-old males and observed no difference between transgenic and wild-type animals (Fig. 9A). It is well established that the production of major urinary proteins is dependent on a normal GH secretion pattern and signaling pathway, and alterations in the abundance of major urinary protein (MUP) in the urine is indicative of perturbed GH production (2, 31, 35, 53). A comparison of transgenic and wild-type mouse urinary MUP levels by SDS-PAGE (Fig. 9B)
indicated that hSTC-2 overexpression had no effect on GH signaling.

IGF-I and IGF-II mRNA levels were examined in transgenic and wild-type tissues, and serum IGF-I was measured in 6-wk-old mice to determine whether hSTC-2 overexpression caused a significant reduction in the production of these growth factors. IGF-II expression is greatest during development. As expected, steady-state IGF-II mRNA levels increased with developmental age, but a significant difference between transgenic and wild-type IGF-II mRNA abundance was not observed (Fig. 9C). Liver IGF-I gene expression was assessed during early postnatal development. As for IGF-II, we did not observe a significant change in IGF-I mRNA levels in transgenics compared with wild-type littermates (Fig. 9D). To confirm the Northern data for IGF-I expression, we measured serum IGF-I by RIA and found higher levels of circulating IGF-I in hSTC-2-transgenic male serum relative to wild-type littermates: 571.2 vs. 355.5 ng/ml, respectively (*P < 0.001). hSTC-2-transgenic female serum IGF-I levels (452.9 ng/ml) were not different from wild-type levels (401.2 ng/ml) (P = 0.3105). Collectively, these data indicate that hSTC-2-induced growth restriction during development and during postnatal life is not likely due to a change in production of GH or the IGFs.

DISCUSSION

The initial objective of our study was to determine whether hSTC-2 would generate phenotypes distinct from those elicited by hSTC-1 in transgenic mice and therein suggest that these factors perform distinct functions in mammals. Our results are the first to show that hSTC-2 is bioactive in mammals and, when constitutively expressed in mice, produces a phenotype similar to that generated by hSTC-1. The primary difference was that serum PO4 and Ca2+ levels in hSTC-1-transgenic mice were significantly altered, whereas we found little evidence for such changes in hSTC-2-transgenic mice. Moreover, high neonate morbidity was caused by hSTC-2 overexpression, whereas this was not reported for hSTC-1-transgenic mice. Collectively, the data show that STC-1 and STC-2 can exert powerful growth-suppressive effects in pre- and postnatal life.

We first determined the expression pattern for the Stc2 gene because previous reports do not agree on the size of the mSTC-2 mRNA or its distribution (8, 18). Our Northern analysis detected 4- and 2-kb STC-2 mRNAs. The 4-kb mRNA was expected because of the long 3′-untranslated sequence (3.1 kb) found in hSTC-2 and mSTC-2 EST sequences. The smaller transcript was likely due to use of alternative polyadenylation signals, because there is no evidence for a larger coding sequence among mSTC-2 EST sequences. Although the expression patterns of the stanniocalcins overlap, the abundance of each mRNA in mature mouse tissues is significantly different. For example, STC-2 is barely detectable in ovary, kidney, and whole embryo RNAs, whereas STC-1 is highly expressed in these tissues (50). This must have important implications for the tissue-specific function of these proteins because, unlike STC-1 in fish, they are not typically found in the circulation. Consequently, their specific sites of
synthesis may be important determinants of their paracrine/autocrine functions.

Among the phenotypic consequences of hSTC-2 overexpression, the first to be observed was the ~30% loss of transgenic neonates in mixed litters, which clearly indicated that hSTC-2 hyperstimulation can be lethal. The physiological basis for transgenic pup mortality was not immediately evident, because transgenic pups did not manifest any obvious physical abnormalities other than being significantly smaller than their wild-type littermates. It is notable that hSTC-1-transgenic mice produced by Filvaroff et al. (13) did not exhibit transgenic neonate morbidity, even though transgene expression was specific to and high in skeletal muscle, similar to the hSTC-2-transgenic mouse model described here. This obviously points

Table 3. Body and tail length comparisons

| Total Length, cm | Body Length, cm | Tail Length, cm | Tail Weight, g | Tail Weight as %Body Weight |
|------------------|-----------------|-----------------|----------------|-----------------------------|
| L314 TgM         | 14.10±0.145*    | 7.778±0.07*     | 6.284±0.075*   | 0.2368±0.009*†             | 1.377±0.044*†               |
| Age-matched wtM  | 17.13±0.147     | 9.383±0.088     | 7.980±0.116    | 0.6155±0.016               | 2.114±0.055                 |
| Weight-matched wtM | 13.57±0.128   | 7.890±0.028     | 6.341±0.211    | 0.4314±0.011               | 2.536±0.057                 |

Values are means ± SE. *P < 0.0001 compared with age-matched wild-type males. †P < 0.0001 compared with weight-matched wild-type males.
Table 4. Overview of absolute organ weights and organ-to-body weight ratios in wild-type and L314 transgenic male mice

|                | 14-wk L314 Male | 14-wk wtM | Weight-Matched wtM |
|----------------|----------------|----------|-------------------|
| Body weight    | 16.51 ± 0.33   | 29.58 ± 0.74 | 16.82 ± 0.22     |
| Brain          | 312.7 ± 5.9    | 420.7 ± 1.3 | 434.2 ± 6.1      |
| %Body weight   | 1.9 ± 0.06†    | 1.42 ± 0.02 | 2.58 ± 0.04      |
| Spleen         | 46.25 ± 3.1    | 11.53 ± 1.8 | 11.54 ± 7.5      |
| %Body weight   | 0.28 ± 0.02‡    | 0.39 ± 0.07 | 0.68 ± 0.04      |
| Kidney         | 382.8 ± 1.2    | 505.5 ± 2.5 | 257.0 ± 6.4      |
| %Body weight   | 2.32 ± 0.06†    | 1.71 ± 0.06 | 1.53 ± 0.04      |
| Lung           | 140.0 ± 5.2    | 204.1 ± 8.5 | 158.3 ± 7.6      |
| %Body weight   | 0.85 ± 0.03*    | 0.69 ± 0.02 | 0.94 ± 0.05      |
| Liver          | 1,112.0 ± 24.3 | 1,494.0 ± 44.5 | 987.0 ± 39.4     |
| %Body weight   | 6.74 ± 0.09†    | 5.06 ± 0.14 | 5.86 ± 0.18      |
| Heart          | 118.4 ± 2.5    | 174.6 ± 7.7 | 110.9 ± 4.2      |
| %Body weight   | 0.72 ± 0.02*    | 0.59 ± 0.03 | 0.66 ± 0.02      |
| Testes (wet)   | 141.8 ± 5.2    | 148.0 ± 9.3 | 78.7 ± 4.8       |
| %Body weight   | 0.86 ± 0.03†    | 0.50 ± 0.03 | 0.47 ± 0.03      |
| Viscera        | 2,560.0 ± 12.0 | 3,916.0 ± 20.0 | 2,219.0 ± 17.0   |
| %Body weight   | 15.45 ± 0.49†   | 13.21 ± 0.54 | 13.18 ± 0.30     |
| Carcass        | 11.41 ± 0.20   | 22.26 ± 0.55 | 12.02 ± 0.13     |
| %Body weight   | 69.18 ± 0.47†   | 75.26 ± 0.25 | 71.47 ± 0.50     |

Table 5. Body content analysis by DEXA

|                | %Lean Mass | %Fat Mass |
|----------------|------------|-----------|
|                | Male       | Female    | Male       | Female    |
| L314 Tg        | 80.82 ± 0.70 (7) | 75.29 ± 0.77 (8) | 19.17 ± 0.67 (7) | 24.78 ± 0.75 (8) |
| Wild type      | 83.33 ± 1.14 (8) | 76.92 ± 0.81 (8) | 16.74 ± 1.14 (8) | 23.29 ± 0.81 (8) |

Fig. 7. hSTC-2-transgenic mice exhibit increased food intake. Calculating the amount of food consumed as %body weight over the 7-day study showed that hSTC-2 transgenics consume more food (22%) than age-matched wild types and less food (17%) than the younger, weight-matched, wild-type mice.
We also examined embryos and neonates for gross morphological indications of developmental delay. The most significant observation related to this is the degree of skull patency in hSTC-2-transgenic pups that in some respects resembles cleidocranial dysplasia (58). This intramembranous bone phenotype was reminiscent of the sagittal and parietal foramen observed in msx2 null mice (19, 40), mice homozygous for the hypomorphic cbfb\(^{GFP}\) allele (21), and mice in which the bone-morphogenic protein antagonist noggin has been applied to the developing cranium (56). In all of these mutant mouse models, the degree of patency along the sagittal and parietal suture lines is significantly greater than in wild-type littermates. Filvaroff et al. (13) found that the cranial suture lines in their adult hSTC-1-transgenic mice were morphologically distinct, and TRAP staining suggested increased osteoclast activity along the cranial suture lines. Interestingly, they also showed that hSTC-1 overexpression decreased the rate at which bone mineralization occurred (13). Collectively, these data point to

![Cranial patency and endochondral ossification. Delay in bone formation exhibited by the hSTC-2-transgenic mice.](image)

Fig. 8. Cranial patency and endochondral ossification. Delay in bone formation exhibited by the hSTC-2-transgenic mice. A: Alizarin red- and Alcian blue-stained hSTC-2 postnatal day 1 skulls revealed a reduction in the amount of bone formation in the transgenic skull (arrows). The space between the wild-type posterior frontal and sagittal sutures is very narrow compared with that found in transgenic mice (black outline). B: pixel area of the space between the edges of the skull bones in the transgenics (●) is more than twice that of wild-type mice (○). C: stained E16.5 wild-type and transgenic embryos from the same litter. *Difference in ossification (red stain) of hip bones (within black circle) and vertebrae.
Obviously, this contributed to the severe postnatal growth which hSTC-1 and -2 exert their effect on bone development. Further studies are currently underway to investigate the mechanism by which the overexpression of either hSTC-1 or hSTC-2 that may alter early developmental delay caused by a decreased rate of bone formation due to the overexpression of either hSTC-1 or hSTC-2. In support of this, our hSTC-2-transgenic embryos showed delayed ossification in different endochondral skeletal elements. Interestingly, these results would not have been predicted on the basis of the recent report from Yoshiko et al. (59) showing that, in vitro, STC-1 actually augments the differentiation of mature rat calvaria osteoblasts shown by the enhanced expression of the osteoblast markers. Therefore, the growth-restrictive properties of STC-1 and STC-2 likely impact other earlier developmental processes and/or cell types.

To begin assessing these possibilities, we performed a series of growth studies with E14.5 MEFs from hSTC-1 and wild-type embryos, because the proliferation rate of MEFs from mouse mutants that exhibit embryonic growth restriction have been successfully replicated in vitro (47, 55, 57). However, experiments with hSTC-1 MEFs did not mimic the growth-restrictive phenotype seen in vivo. Results similar to ours were obtained using MEFs prepared from p57Kip2-deficient mice that also exhibit skeletal abnormalities and growth retardation (44) and with MEFs from cdk4 null mice that display growth retardation to the same extent as our transgenic mice (48). The lack of a proliferation defect in hSTC-1-transgenic MEFs suggests that the reduced-growth phenotype is restricted to a different tissue compartment during development and that these effects are cumulative, causing a progressive and proportional reduction in embryo mass.

Many mouse mutants exhibiting a dwarf phenotype are the result of disrupted signaling of the GH/IGF pathway (9, 15, 25, 36). Changes in the levels of GH/IGF signaling components can also occur secondary to the primary mutation and result in growth restriction (3, 14, 20, 22). However, as in the case of hSTC-1 overexpression (49), we did not detect changes in GH/IGF signaling in response to hSTC-2 overexpression, leading us to conclude that intrauterine and postnatal growth retardation occurred independently of this pathway. Clearly, our data indicate that an intact IGF-I signaling system restriction seen in our hSTC-1 and hSTC-2-transgenic mice. However, our data also show that growth retardation occurs early in development, before significant bone ossification begins. Therefore, the growth-restrictive properties of STC-1 and STC-2 likely impact other earlier developmental processes and/or cell types.

Table 6. Serum calcium, phosphate, and alkaline phosphatase levels in hSTC-2-transgenic and wild-type mice

|          | Calcium, mmol/l | Phosphate, mmol/l | Alkaline Phosphatase, U/l |
|----------|-----------------|-------------------|--------------------------|
| L314     |                 |                   |                          |
| wtM      | 2.836±0.030     | 3.323±0.079       | 132.1±7.203              |
| TgM      | 2.849±0.044     | 3.311±0.126       | 107.5±3.612*             |
| wtF      | 2.840±0.027     | 3.141±0.068       | 159.7±6.977              |
| TgF      | 2.765±0.021*    | 3.347±0.013       | 133.9±7.974*             |
| L372     |                 |                   |                          |
| wtM      | 2.796±0.029     | 3.406±0.111       | 122.6±8.752              |
| TgM      | 2.769±0.035     | 3.457±0.073       | 108.9±8.221              |
| wtF      | 2.759±0.029     | 3.232±0.01        | 132.1±6.579              |
| TgF      | 2.678±0.027     | 3.481±0.078       | 144.8±11.77              |

Values represent means ± SE of 10–15 mice with multiple parameters measured from each mouse. *P < 0.05 compared with sex-matched wild types of the same line.

Fig. 9. Assessment of the somatomedin pathway. A: growth hormone (GH) mRNA expression was analyzed by Northern blot using 5 μg of pooled total pituitary RNA, and steady-state mRNA levels were not different between wild types (wtM) and transgenics (TgM) or both lines. B: downstream GH signaling was assessed by Coomassie-stained SDS-PAGE of transgenic and wild-type urine for major urinary protein (MUP). The level of MUP was not decreased in the transgenics compared with wild types in both sexes. C: embryonic IGF-II mRNA expression, determined by Northern blotting of whole embryo total RNA (30 μg), showed no difference in its expression between transgenics and wild types. D: postnatal IGF-I Northern analysis also showed no observable difference in expression levels in liver total RNA (30 μg).
cannot counteract the effects of excess circulating levels of hSTC-2.

Recently, McCudden et al. (29) showed that STC-1 and its receptor can localize to mitochondria in a variety of tissues and that STC-1 can increase the respiration rate of isolated liver mitochondria in vitro. It is possible that constitutively elevated hSTC-2 resulted in altered metabolism and that this had a contributing effect on the growth-restrictive phenotype. Food consumption by the hSTC-2-transgenic mice was 22% greater than age-matched wild-type mice. Filvaroff et al. (13) also found a comparable increase in food intake along with a 10% increase in oxygen consumption in their hSTC-1-transgenic mice. Obviously, hyperphagia, in combination with increased oxygen requirement, is highly suggestive of a metabolic defect and appears to be a hallmark of excess circulating hSTC-1 or hSTC-2. Hyperphagia has been documented in a number of growth-restricted mouse mutants and is usually associated with aberrant expression or signaling of proteins that regulate various aspects of metabolism, including GH (10), melanocortin-1 and -4 receptors (17, 27), and fibroblast growth factor-19 (46). These data are consistent with a role for stanniocalcins in controlling certain aspects of metabolism.

On the basis of the data presented here, we propose that the stanniocalcins represent a new class of trophic factors that play a role in growth regulation independently of the GH/IGF axis. When present in supraphysiological levels, stanniocalcins cause developmental delay, especially in intramembranous bone. Our findings to date, along with those of Yoshiko et al. (59), advocate a role for stanniocalcins in regulating bone development. The fact that hSTC-2 overexpression is lethal to a proportion of neonates implies that this hormone has deleterious effects on mammalian development that are linked to cell proliferation and differentiation. The precise mechanism by which stanniocalcins reduce growth and cause death is critical to our understanding of their importance in mammalian physiology, and it appears that tissue-specific effects on cellular metabolism may be key to their mechanism of action.

ACKNOWLEDGMENTS

We thank Dr. Chris Pin for critical reading and constructive comments on the manuscript.

GRANTS

This research was supported by an operating grant to G. E. DiMattia from the Canadian Institutes of Health Research (CIHR) and a grant from the Lawson Health Research Institute Internal Research Fund. A. D. Gagliardi was supported by a student fellowship from the CIHR Strategic Training Program and a grant from the Lawson Health Research Institute Internal Research Fund. A. D. Gagliardi was the Canadian Institutes of Health Research (CIHR) and a grant from the Lawson Health Research Institute Internal Research Fund.

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