The p53 family member p73 modulates the proproliferative role of IGFBP3 in short children born small for gestational age

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ABSTRACT **The regulation of insulin-like growth factor–binding protein 3 (IGFBP3) gene expression is complex, because it can be induced by agents that both stimulate and inhibit the proliferation. The principal aim of this study was to investigate whether p73, a member of the p53 gene family, has a role in the regulation of the IGFBP3 expression and whether this regulation occurs in a context of cell survival or death. We demonstrate that IGFBP3 is a direct TAp73α (the p73 isoform that contains the *trans*-activation domain) target gene and activates the expression of IGFBP3 in actively proliferating cells. As IGFBP3 plays a key role in regulating the growth hormone/insulin-like growth factor type 1 (GH/IGF1) axis, whose alterations in gene expression appear to have a role in the growth failure of children born small for gestational age (SGA), we measured the mRNA expression levels of p73 and IGFBP3 in a group of SGA children. We found that mRNA expression levels of p73 and IGFBP3 are significantly lower in SGA children compared with controls and, in particular, p73 mRNA expression is significantly lower in SGA children with respect to height. Our results shed light on the intricate GH/IGF pathway, suggesting p73 as a good biomarker of the clinical risk for SGA children to remain short in adulthood.**

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INTRODUCTION

Insulin-like growth factor–binding protein 3 (IGFBP3), the main circulating carrier of insulin-like growth factors (IGFs) in postnatal life, inhibits or enhances the effects of IGFs by modulating the amount of free IGF type 1 (IGF-1), by repressing its transfer from the circulation to tissue sites of action, and by regulating the interaction between IGFs and IGF-1 receptor (IGF-1R) (Mohan and Baylink, 2002). The growth hormone (GH)/IGF/IGFBP axis has been extensively studied in neonates with intrauterine growth retardation and in children born small for gestational age (SGA).

SGA children represent a group of newborns (5–10%) whose birth weight and/or length are less than −2 SD from the mean for gestational age (Clayton *et al.*, 2007). Most SGA children show catch-up growth during the first 2 yr of life, but ~15% remain short throughout childhood and adolescence and into adulthood (Klammt *et al.*, 2008; Labarta *et al.*, 2009). The status of the GH/IGF axis at birth or in early postnatal life is not predictive of later growth, and therefore hormone measurements in SGA infants or children are not indicated in routine care (Leger *et al.*, 1996). Aside from its effects on IGF-1/IGF-1R interaction, IGFBP3 also plays a role as growth inhibitor and proapoptotic factor in an IGF-independent

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Abbreviations used: AD, Alzheimer's disease; AGA, appropriate for gestational age; ChIP, chromatin immunoprecipitation; GH, growth hormone; IGF, insulin-like growth factor; IGF-1, IGF type 1; IGF-1R, IGF-1 receptor; IGFBP3, insulin-like growth factor–binding protein 3; PI, propidium iodide; RE, responsive element; SGA, small for gestational age; shRNA, short hairpin RNA.

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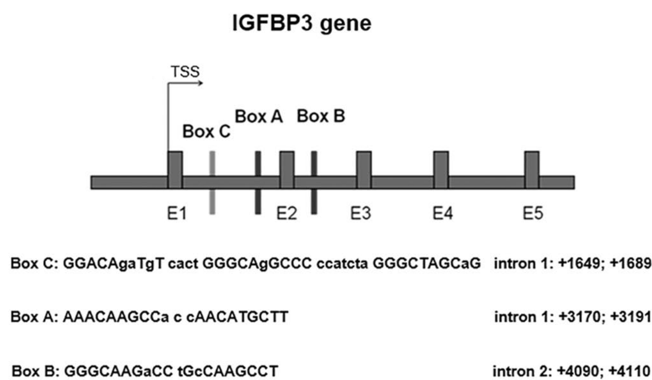


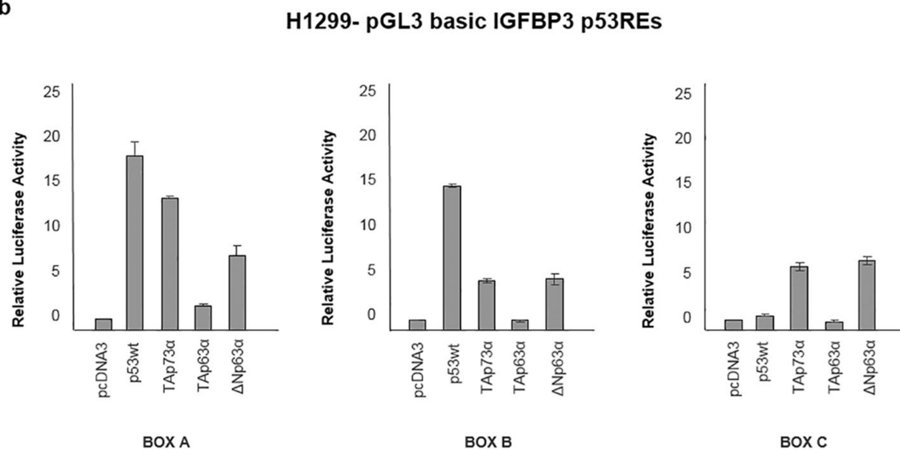


FIGURE 1: (a) Schematic map of the human *IGFBP3* genomic region containing the two known p53REs (box A and box B) and the new p53RE (box C) with related sequences. (b) Luciferase assay. Effect of p53, TAp73α, TAp63α, and ΔNp63α on the transcriptional activity of IGFBP3-p53REs. p53-null H1299 cells were transiently cotransfected with pcDNA3 empty vector or expressing p53, TAp73α, TAp63α, or ΔNp63α and pGL3-Basic-IGFBP3-p53RE-box A, B, or C. The luciferase activities were normalized to *Renilla* activity. The data represent the average of at least three independent experiments and are shown with SEs.

manner (Firth and Baxter, 2002; Longobardi *et al.*, 2003; Takaoka *et al.*, 2006). Considering that IGFBP3 plays a role in several different contexts, its expression is regulated by many factors. In a previous study, we demonstrated that some polymorphisms in the *IGFBP3* promoter region, such as –667 G/A and –396 C/T, influence the basal transcriptional activity of the *IGFBP3* gene in SGA children, although IGFBP3 expression remains in the normal range for age and sex (Faienza *et al.*, 2011). GH and insulin regulate the proproliferative role of IGFBP3 (Liao *et al.*, 2006; DiGirolamo *et al.*, 2007); proapoptotic and growth inhibitor factors, such as transforming growth factor-β, retinoic acid, tumor necrosis factor-α, vitamin D, antiestrogens, antiandrogens, and tumor suppressor p53, stimulate IGFBP3 expression (Huynh *et al.*, 1996; Nickerson and Huynh, 1999). It is already known that the *IGFBP3* gene is a direct target of p53, actively participating in apoptotic pathways triggered by p53. Historically, p53 represents the founding member of a transcription factor family to which also p63 and p73 belong. The three transcriptional factors share common functional characteristics, including the ability to induce cell cycle arrest and apoptosis, but have nonredundant roles (Vousden and Ryan, 2009; Gottlieb and Vousden, 2010). The three genes produce different protein isoforms that are generated through the use of two promoters and alternative splicing events. The TA isoforms (with the *trans*-activation domain) have anti-oncogenic activity, whereas the ΔN isoforms (without the *trans*-activation domain) have a potential anti-apoptotic and proproliferative function (Murray-Zmijewski *et al.*, 2006; Deyoung and Ellisen, 2007). It has been reported that ΔNp63α down-regulates IGFBP3 expression (Barbieri *et al.*, 2005), but the role of p73 in the regulation of IGFBP3 expression has not yet been determined.

As the p53 family members are central regulators of cell cycle arrest, apoptosis, and proliferation, and given the dual functional role of IGFBP3, the principal aim of this study was to investigate whether TAp73 has a role in the regulation of the IGFBP3 expression and whether this regulation occurs in a context of cell survival or death. Consistently, we measured the serum expression levels of TAp73 and IGFBP3 in a group of short children born SGA and in a control group born appropriate for gestational age (AGA) to assess the outcome of TAp73 and IGFBP3 measurements in the clinical risk for SGA babies to remain short in adulthood.

RESULTS

TAp73α activates the transcription of *IGFBP3*

It is already known that the *IGFBP3* gene is a direct target of p53 and contains two p53 responsive elements (REs) located in intron 1 and intron 2: box A and box B, respectively. We identified, upstream and closer to the transcription start site, the already identified p53RE box A and box B (Barbieri *et al.*, 2005) and an additional p53RE in intron 1, which we called box C (Figure 1). To determine whether p53 family members can transactivate *IGFBP3* gene expression via this new element, we cotransfected the p53-null H1299 cells with pGL3-basic-IGFBP3-box A, pGL3-basic-IGFBP3-box B, or pGL3-basic-IGFBP3-box C and the recombinant vectors expressing either the human p53, TAp73α, TAp63α, and ΔNp63α or their DNA binding–deficient mutants (Figure 1 and Supplemental Figure 1). Reporter assay experiments demonstrated that, although with different efficiencies, box A and box B were transactivated by p53. Interestingly, we found that, under these conditions, TAp73α and ΔNp63α also activated the expression of the reporter gene via these two p53REs (Figure 1). Surprisingly, the p53RE we identified (box C) was exclusively activated again by TAp73α and ΔNp63α and not by p53 (Figure 1). The activation was dependent on functional p53, p63, and TAp73α, since transactivation with the defective mutants p53R175H, TAp73αV156A, TAp63αR279Q, or ΔNp63αR279Q was incapable of activating the reporter constructs (Supplemental Figure 1). These results suggested that the p53 family members regulate the expression of IGFBP3 by interacting differentially with the three p53REs present in the gene. Next we tested the effect of p53 family member overexpression on endogenous *IGFBP3* gene expression in p53 wild-type and null background.

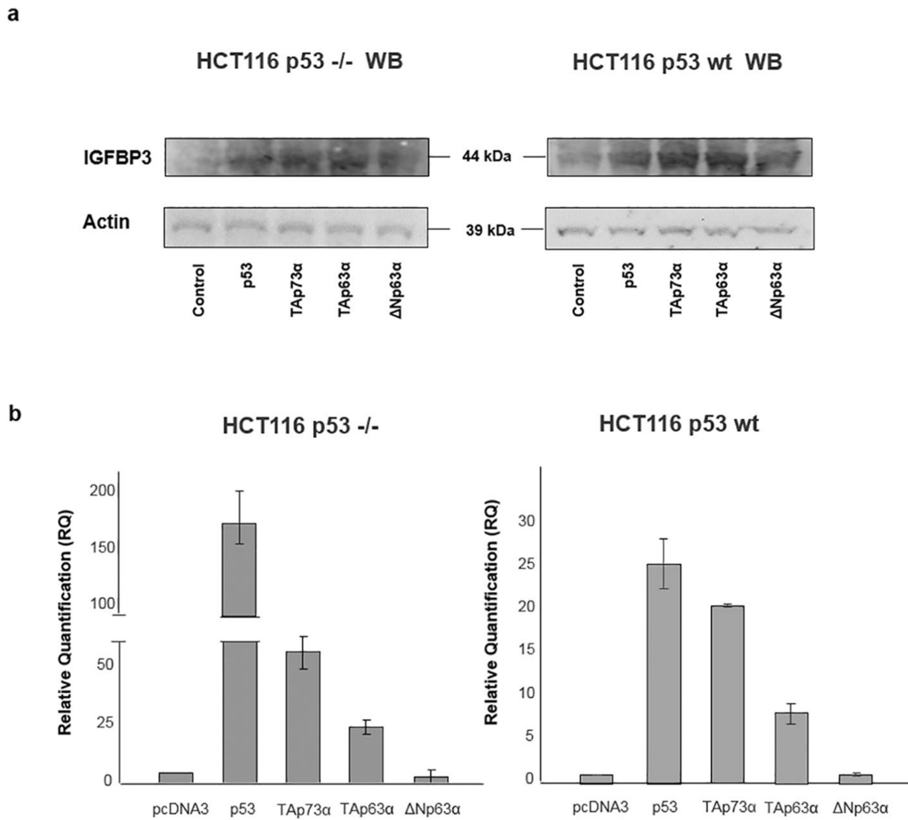


FIGURE 2: (a) Western blotting of IGFBP3 protein in HCT116-p53$^{-/-}$ and HCT116-p53^{wt} cells transfected with pcDNA3 control vector, pcDNA3-p53, pcDNA3-TAp73α, pcDNA3-TAp63α, or pcDNA3-ΔNp63α. Western blotting of actin was conducted as a control. (b) RT-qPCR of IGFBP3-mRNA in HCT116-p53$^{-/-}$ and HCT116-p53^{wt} cells transfected with pcDNA3 control vector, pcDNA3-p53, pcDNA3-TAp73α, pcDNA3-TAp63α, or pcDNA3-ΔNp63α. The average expression (± SD) is normalized to GAPDH expression level.

We transiently transfected the human isogenic colon cancer cell lines HCT116-p53wt and HCT116-p53-null with p53, TAp73α, TAp63α, and ΔNp63α proteins. We monitored the overexpression of the proteins 24 h after transfection by Western blot analysis (Supplemental Figure 2). Western blotting and quantitative real time-PCR (qRT-PCR) experiments demonstrated that IGFBP3 mRNA and protein were induced by p53, TAp73α, and TAp63α but not by ΔNp63α, independently by p53 background (Figure 2, a and b). The TAp73α-dependent IGFBP3 expression was also demonstrated in other p53wt or null cell lines (Supplemental Figure 3). Although ΔNp63α activated the IGFBP3 expression through boxes A, B, and C in the reporter assays (Figure 1), in the HCT116 cell line, ΔNp63α weakly induced the expression of endogenous *IGFBP3* gene, as previously reported (Barbieri *et al.*, 2005). Altogether these experiments demonstrated that *IGFBP3* gene is a TAp73α transcriptional target as well as a p53 target.

TAp73α binds boxes A, B, and C in the *IGFBP3* gene, and p73 suppression leads to IGFBP3 down-regulation

We tested the ability of TAp73α to bind the three p53REs in the *IGFBP3* gene by performing chromatin immunoprecipitation (ChIP) experiments. The cross-linked chromatin from isogenic human embryonic kidney Flp-In T-Rex-293 expressing p53 and TAp73α, at 24 h after the induction of the two proteins, was immunoprecipitated with the respective antibodies or without antibody as negative control. We found that p53 and TAp73α were consistently recruited on the IGFBP3 box A. The p53 and TAp73α binding was accompanied by an increase in histone H4 acetylation (Figure 3a). Surprisingly, only TAp73α but not p53 was bound to IGFBP3 box B and box C (Figure 3a). Interestingly, TAp73α binding on boxes A, B, and C was accompanied by histone H4 acetylation, which suggested that TAp73α actively transcribes IGFBP3. At the same time, we examined whether TAp73α can regulate the expression of the endogenous IGFBP3 gene. Total RNA and RNA extracted from T-Rex-293-TAp73α cells, both uninduced and at 24 h after induction of TAp73α expression, were analyzed in RT-qPCR experiments and Western blotting. We clearly demonstrated that TAp73α induced the expression of IGFBP3 mRNA with great efficiency (Figure 3b). In addition, to confirm the contribution of p73 protein in the transcriptional regulation of the *IGFBP3* endogenous gene, we selectively suppressed the expression of p73 by transfecting specific p73 short hairpin RNAs (p73-shRNAs) in MCF-7 cells (Figure 4a). The p73-shRNAs we used are designed to target all known p73 variants without targeting related gene family members. Cells were transfected with pRS empty vector (control) or p73-shRNAs for 48 h. As shown in Figure 4a, RT-qPCR and Western blot analyses showed that the cells transfected with p73-shRNA displayed a decrease in the levels of their mRNAs and corresponding proteins. At the same time, in cells that underwent suppression of p73, a marked decrease was observed in IGFBP3 mRNA, further supporting the concept that IGFBP3 is also a p73 target gene (Figure 4b).

TAp73α activates IGFBP3 expression during cell cycle progression

As previously reported, TAp73α protein levels undergo a coordinated and complex modulation during physiologic cell cycle progression, reaching a peak in S phase, and their silencing determines a significant suppression of proliferation compared with the control (Figure 5, a and b; Talos *et al.*, 2010). We therefore sought to correlate the transcription of IGFBP3 in arrested and serum-released MCF-7 cells. Cells were grown in low serum for 48 h and were released by serum restimulation for 22 h. The cell cycle profile was monitored by flow-cytometric analyses (Figure 5a) and by immunoblots of cyclins D1, E, and A (Figure 5b), which indicated that MCF-7 cells were arrested in G_1 after 48 h of serum starvation and were in S phase 22 h after the refeed. As shown in Figure 5b, TAp73α fell as the cells accumulated in G_0/G_1 and increased after re-addition of serum, reaching a peak when the cells were in S phase (22 h; Lefkimmiatis *et al.*, 2009). We confirmed that the suppression of p73 in asynchronous and reefed MCF-7 cells led to a reduction in cell proliferation (the percentage of cells in S+G_2 phases decreased). To assess whether the changes we observed in p73 protein levels at G_1 and S phases affected the expression of IGFBP3, we performed RT-qPCR (Figure 5c). Interestingly, we found that in G_0/G_1-arrested cells, when TAp73α protein levels were low, the IGFBP3 expression

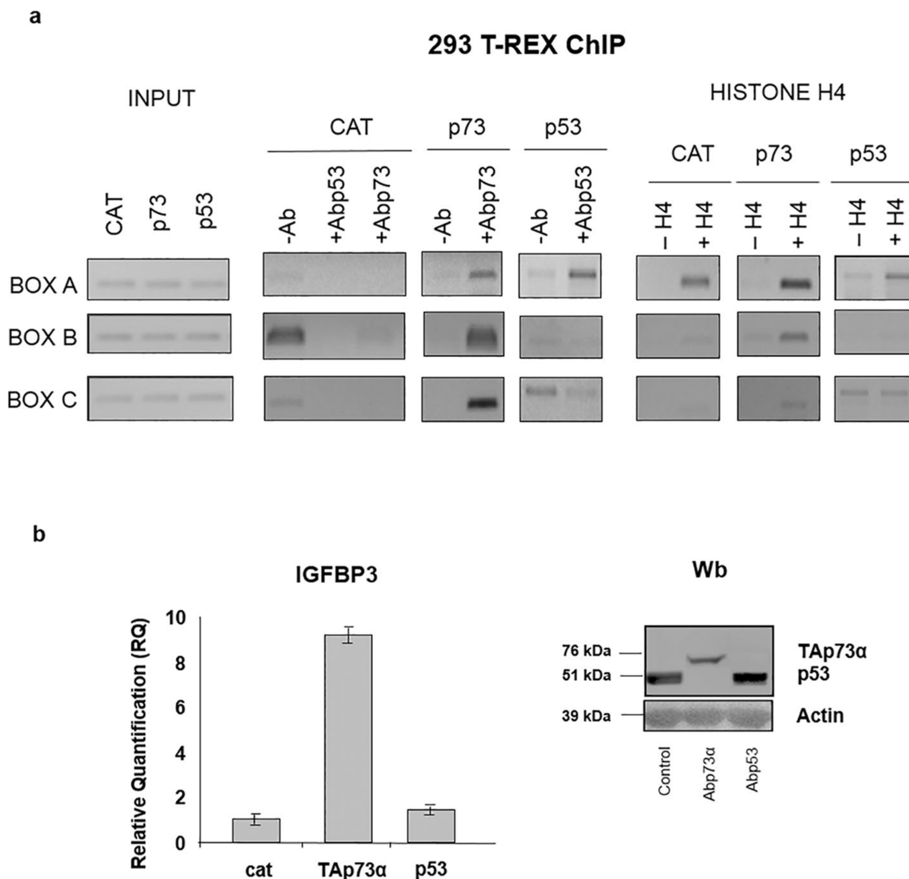


FIGURE 3: (a) In vivo recruitment of p73, p53, and acetylated H4 histone to p53REs boxes A, B, and C present in the *IGFBP3* gene by ChIP assay. Cross-linked chromatin was extracted from the Flp-In-T-Rex 293 cells expressing p53 and TAp73α and immunoprecipitated with the indicated antibodies. The immunoprecipitated material was amplified using primers specific for IGFBP3-box A, -box B, and -box C. Primers specific for unrelated interlukin-10 promoter were used as a negative control. (b) mRNA IGFBP3 expression levels were detected by RT-qPCR, and the exogenous overexpressed p53 and p73 proteins were detected by Western blotting.

was decreased compared with refed and asynchronous growing cells when TAp73α protein levels are higher. Moreover, the suppression of p73 protein expression by specific p73-shRNAs was paralleled by a further decrease in IGFBP3 mRNA levels both in asynchronous and in starved and refed cells (Figure 5c). All these data strongly suggest that TAp73α, in contrast to p53, regulates the expression of IGFBP3 in actively proliferating cells.

p73 and IGFBP3 expression are down-regulated in SGA children

As IGFBP3 plays a key role in regulating the GH/IGF axis, alterations of which in gene expression appear to have a basic role in the growth failure of children born SGA, we assessed the relative contribution of p73 in regulating IGFBP3 expression in SGA children compared with age- and sex-matched AGA controls. Sixteen SGA children and 14 AGA children were analyzed for IGFBP3 and p73 expression by RT-qPCR. Interestingly, as shown in Figure 6a, on average, SGA children expressed both serum p73 mRNAs and IGFBP3 at lower levels than AGA controls (34 and 55% less, respectively; p value = 0.15 and p value = 0.02). Interestingly, we found that p73 expression is significantly lower in SGA children with respect to height compared with AGA children (62% less; p value = 0.031), while IGFBP3 expression is equally lower in SGA children with respect to height and weight (67% less, compared with AGA children; p values = 0.009 and 0.07, respectively). Consistently, the circulating IGFBP3 protein levels in children born SGA with respect to height were lower compared with control values (2076 ng/ml vs. 3648 p value = 0.0002; Figure 6b). We found that p53 mRNA expression was lower in SGA children compared with AGA children, but this difference was not statistically significant (unpublished data).

Altogether these experiments suggest that p73 and IGFBP3 are down-regulated in SGA children.

DISCUSSION

Short stature, with an incidence of three in 100, is a fairly frequent disorder in children. The child with short stature presents a diagnostic puzzle in which the clinical phenotype, endocrine features, and genetic factors may all contribute to the categorization of the patient into a specific diagnostic group. Mutations in different genes, such as *SHOX* (short stature homeobox), *PTPN11*, *RAS*, and *RAF1*, have been shown to be associated with the short stature phenotype in patients with differing pathologies.

Persistent short stature is also one of the most frequent complications of being born SGA, as almost 10–15% of such children have a low adult height. Although the pathophysiology of postnatal growth failure in SGA is complex, in contrast to the above-mentioned short statures due to mutations in different genes, mutations or deletions in the genes involved in GH/IGFs/IGFBP3 axis were rarely found. Therefore a detailed knowledge of gene expression profiling may provide crucial elements to clarify the molecular mechanisms that regulate the persistence of short stature in adults born SGA.

This study addresses the question of whether the p53 family member TAp73α may have a role in cell proliferation via mechanisms that involve regulation of *IGFBP3* gene expression with the aim of shedding light on the molecular mechanisms governing the GH/IGFs/IGFBP3 axis.

We demonstrate here that *IGFBP3* gene expression is promoted by TAp73α as well as by p53 protein. Indeed, we identified a new p53RE in the *IGFBP3* gene, which we called box C. This box is selectively bound and transactivated by TAp73α but not by p53. Many factors influence the ability of p53 to function as a sequence-specific transcription factor, including cofactors that may be stress specific, the variation in p53 levels in response to stress, and the specific RE sequence. p53 binding affinities seem to dictate the choice between regulating cell cycle arrest (high-affinity sites) and proapoptotic responses (low-affinity sites). High-affinity p53 binding sites are characterized by a short spacer between the two decamers and by the arrangement of the consensus CWWG core. Indeed, altering C or G in either decamer can dramatically reduce the responsiveness to p53, and the arrangement CATG allows much stronger transactivations than the other three possibilities (CAAG, CTAG, or CTTG; Menendez *et al.*, 2009). Much less is known about the regulation of target genes by the two p53 relatives, p63 and p73, which is

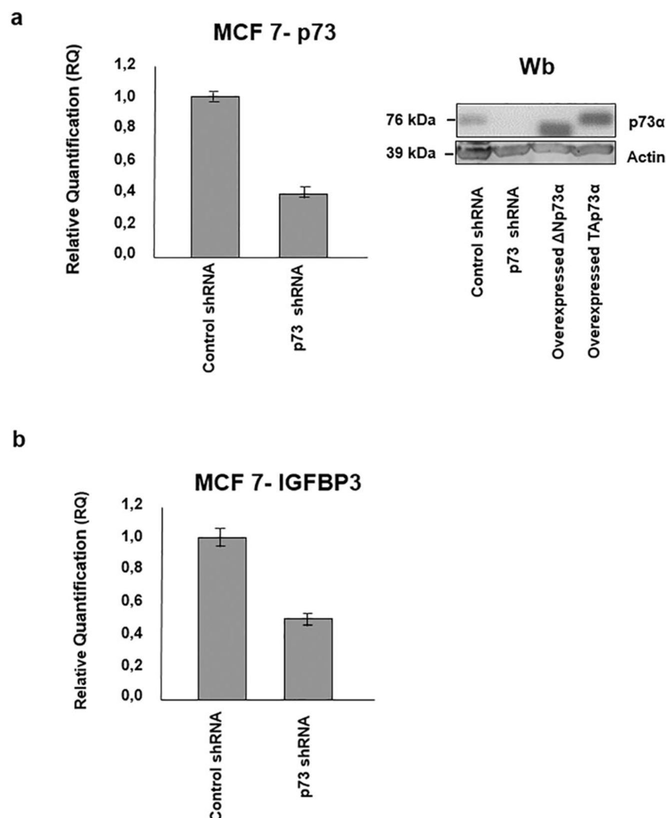


FIGURE 4: (a) The suppression of endogenous p73 mRNA and protein was evaluated by RT-qPCR and Western blotting. (b) IGFBP3 RT-qPCR in MCF-7 cell line transfected with unspecific shRNAs (pRS) or with four specific p73 shRNAs.

achieved through binding to sequence-specific REs whose consensus sequences are highly similar to that of p53 REs. Among the three REs in the *IGFBP3* gene, p53 binds box A with high affinity. This latter RE is composed of two decamers spaced by only one base, and moreover, it is the only one that has the more strongly arranged CATG consensus core sequence, which might explain the p53 binding specificity.

Up to now, the regulation of *IGFBP3* gene expression by the p53 family members has been linked to the apoptotic, antiproliferative functions (Buckbinder *et al.*, 1995; Barbieri *et al.*, 2005), although the exact mechanisms underlying cell cycle arrest and apoptosis by IGFBP3 have yet to be fully elucidated. p73 has a significant role in brain development and in both embryonic and adult neurogenesis. p73 has a critical role in maintaining an adequate neurogenic pool by promoting self-renewal and proliferation (Meyer *et al.*, 2004; Talos *et al.*, 2010; Niklison-Chirou *et al.*, 2013). Interestingly, a decrease in IGFBP3 expression has been observed in an Alzheimer's disease (AD) model cell line and in the hippocampus of AD model transgenic mice (Talos *et al.*, 2010), indicating a protective role for IGFBP3 in primary rat hippocampal neurons. Importantly, IGFBP3 can also be involved in neuronal survival and protection via IGF-1–independent mechanisms. In contrast to reports of the proapoptotic effects of IGFBP3, a number of recent studies demonstrated that IGFBP3 stimulates cell proliferation and protects cells from apoptotic insults in a variety of cell types through IGF-dependent or IGF-independent mechanisms (Martin *et al.*, 2003; Butt *et al.*, 2004; Sung *et al.*, 2014). Of note, we found that the IGFBP3 expression is lower in G_0/G_1-arrested cells and increases when the cells are refed and that the suppression of p73 by using specific p73sh-RNA decreases IGFBP3 expression (Figure 5c). These data strongly suggest that p73 activates the expression of the IGFBP3 in actively proliferating cells.

Several studies provided evidence that p53 family members have broader roles in cell cycle control than previously expected, and we have previously demonstrated a role for p73 and p63 in supporting cellular proliferation through the transcriptional activation of the genes involved in G_1/S and G_2/M progression (Lefkimmiatis *et al.*, 2009). Moreover, TAp73α is implicated in the control of mitosis and aneuploidy (Fulco *et al.*, 2003; Merlo *et al.*, 2005; Talos *et al.*, 2007), and more recently it has been reported that TAp73α has a crucial role in preventing genomic instability in multiple tissues (Tomasini *et al.*, 2009; Costanzo *et al.*, 2014).

As a growing number of studies indicate that alterations of the genes involved in the signal transduction pathways of GH/IGF/IGFBP3 play a key role in short children who do not respond to GH treatment, we assessed the contribution of p73 in regulating the expression of IGFBP3 in a group of children born SGA. Of note, SGA children are at increased risk for neurodevelopmental disorders, and p73, as reported above, has an important role in brain development. Measuring the mRNA expression levels of IGFBP3 and p73 in children born SGA and in a group of AGA controls, we found that the mRNA expression levels of p73 and IGFBP3 are consistently significantly lower in SGA children compared with AGA controls, and p73 mRNA expression in particular is significantly lower in short children born SGA with respect to height. Of note, we observed in the same children that the circulating IGFBP3 protein levels were lower compared with control values (Figure 6, a and b).

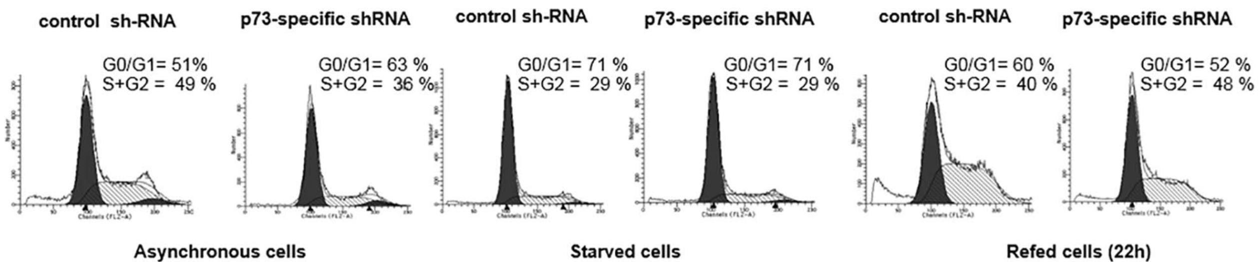
Altogether our experiments demonstrate for the first time a functional link between IGFBP3 and TAp73α and strongly support the hypothesis that the measurement of circulating levels of IGFBP3 in association with analysis of the expression of p73 could allow the identification of patients at risk to remain short in adulthood. This finding is particularly important, since there is no predictive biomarker for this state to date.

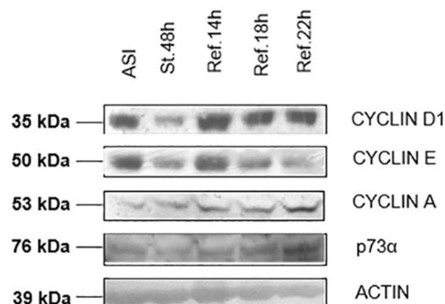
MATERIALS AND METHODS

Subjects

Sixteen children (10 males; mean age 9.08 ± 3.87 yr) out of 20 meeting the inclusion criteria agreed to participate to this study. All children were born at term at the Neonatal Intensive Care Unit (NICU) of the Department of Biomedical Sciences and Human Oncology, Section of Pediatrics, University of Bari, and had a birth length and/or birth weight below −2 SD, height below −2 SD, and no growth failure caused by other disorders (i.e., GH deficiency, metabolic or endocrine diseases). They were followed up for the first 2 yr of life at the Pediatric Endocrinology Unit according to an established follow-up in SGA subjects and were subsequently invited to participate in the study in the period between January and October 2014. The control group consisted of 14 children born AGA (11 males; mean age 9.53 ± 3.62 yr) with birth length and weight above −1 SD according to the Italian Neonatal Anthropometric Charts (Bertino *et al.*, 2010), recruited from patients attending the pediatric clinic of the University of Bari for minor trauma (first aid) or for allergic screening. Written informed consent was obtained from all parents, and oral consent was obtained from all children. All the procedures used were in accordance with the guidelines of the Helsinki Declaration on Human Experimentation.

Table 1 shows the clinical and biochemical characteristics of the SGA and AGA groups.





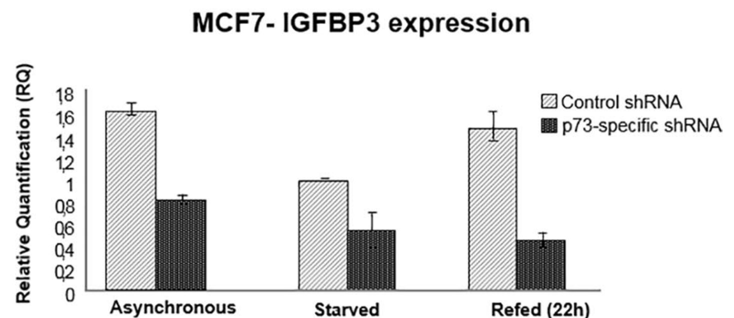


FIGURE 5: (a) The DNA content was quantified in flow-cytometric analysis by PI staining of fixed asynchronous, serum-starved, and refed MCF-7 cells that were treated with unspecific shRNAs (control) or four different p73 shRNAs (Origene), and analyzed in a FACScalibur with CellQuest analysis software (Becton Dickinson). The cell cycle analysis was performed with ModFit software (Becton Dickinson). (b) The cell cycle profile of MCF-7 cells was monitored by immunoblot of cyclins D1, E, and A. Western blot analysis of TAp73α, in asynchronous, serum-starved, and refed MCF-7 cells was conducted at the same time points. The level of actin was evaluated as a control. (c) The relative quantification of IGFBP3 mRNA levels was evaluated by RT-qPCR in asynchronous, serum-starved, and refed MCF-7 cells treated with the unspecific shRNAs (control) or four different p73 shRNAs (Origene). The data represent the average of three independent experiments and are shown with SEs.

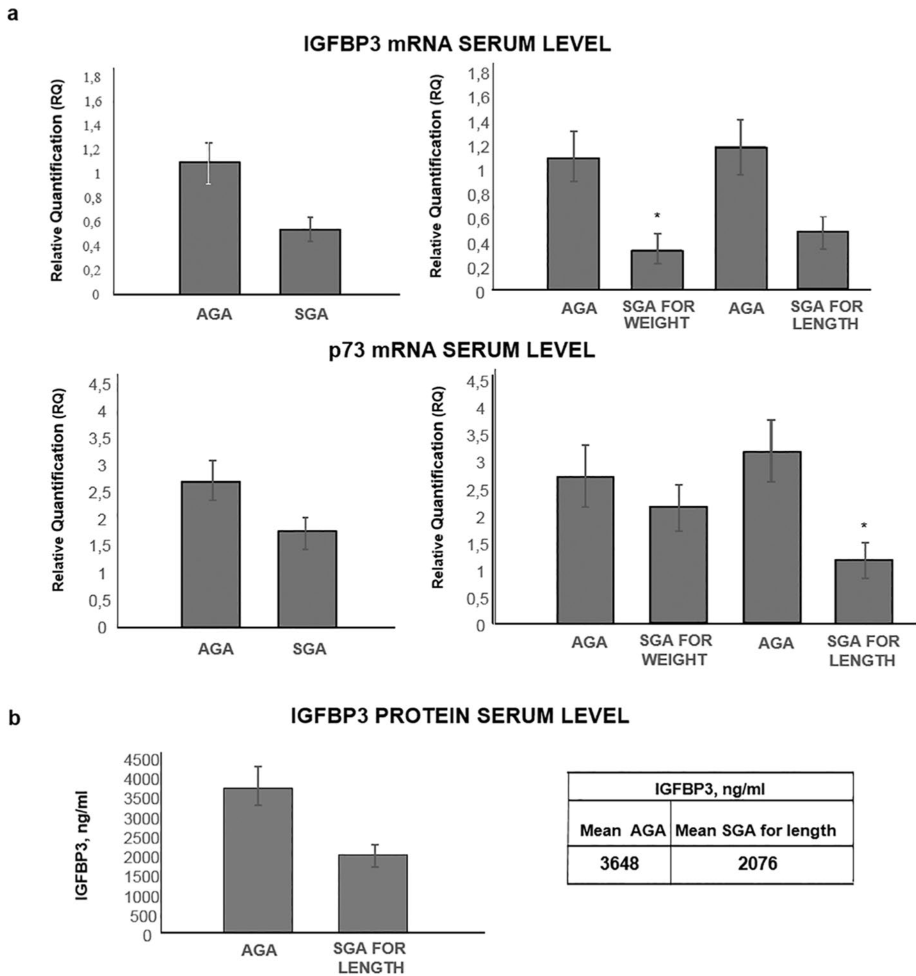
Serum IGFBP3 and IGF-I detection. The serum levels of IGFBP3 from patients were measured by using enzymatically amplified a two-step sandwich-type immunoassay DRG IGFBP-3 (EIA-3300) ELISA kit (DRG Instruments GmbH, Marburg, Germany). Serum levels of IGF-I were measured by the Immulite 2000 IGF-I kit (Siemens Healthcare, Malvern, PA), which is a solid-phase enzyme-labeled chemiluminescent immunometric assay. The IGFBP3 and IGF-1 reference values were taken from the Pediatric Reference Ranges supplied by the manuals of the DRG IGFBP-3 (EIA-3300) ELISA kit (DRG Instruments GmbH) and the Immulite 2000 IGF-I kit.

p53REs identification in the IGFBP3 regulatory regions. The computational analysis to identify the p53REs in the IGFBP3 regulatory regions was performed using the PatSearch algorithm implemented in the DNAfan tool (Grillo *et al*., 2003; Gisel *et al*., 2004; Sbisà *et al*., 2007). Using the p53RE syntax pattern previously described (Grillo *et al*., 2003; Sbisà *et al*., 2007), we searched for p53REs presence in the IGFBP3 promoter or 5′ UTR or 3′ UTR or in the intronic regions, and we found a new p53RE (named box C: +1649–1689), upstream of the two previously identified p53REs (box A: +3170–3191; box B: +4090–4110; NC_000007.14; Buckbinder *et al*., 1995).

Cell lines. The human colon carcinoma cell lines HCT116 and HCT116p53$^{-/-}$, the human breast carcinoma cell line MCF-7, the human lung carcinoma cell line H1299, and the embryonic kidney T-Rex-293 were cultured in DMEM plus 10% fetal bovine serum, L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 µg/ml) at 37°C, 5% CO_2. The embryonic kidney Flp-In T-Rex-293 cell line from Invitrogen was used to generate stable CAT (chloramphenicol acetyltransferase), p53, and TAp73α expression.

Transfections. Cells (5×10^5) were plated 24 h before transfection. At the time of transfection (60–80% cell confluence), 200 µl of DMEM without serum was incubated with Trans-LT1 Mirus transfection reagent (Tema Ricerca, Bologna, Italy) for 5 min at room temperature. Then the empty $pcDNA_3$ vector (control), $pcDNA_3$-p53wt, $pcDNA_3$-p73α, $pcDNA_3$-TAp63α, $pcDNA_3$-ΔNp63α, the empty pRS (control), or four different p73 shRNAs (Origene, Rockville, MD) were added to the medium containing the transfection reagent and incubated at room temperature for 20 min and subsequently added to the cell cultures for the indicated times.

Protein extraction and Western blot analysis. Cells were plated in 100-mm culture dishes at a density of 1×10^6 cells/ml. After treatment, cells were lysed and extracted as previously described (Tullo *et al*., 2003). For immunoblotting, the following primary antibodies were used: p53-specific DO-1 (Santa Cruz Biotechnology, Dallas, TX; 1:300), p63 antibody 4A4 (Santa Cruz Biotechnology;



IGFBP3, ng/ml	
Mean AGA	Mean SGA for length
3648	2076

FIGURE 6: (a) RT-qPCR of IGFBP3 and p73 mRNA expression in SGA children. Expression data were normalized by HPRT1 expression and measured with respect to one normal sample chosen arbitrarily as a calibrator. The reported data represent the average of at least three independent experiments and are shown with their SEs. *, p value < 0.05. (b) IGFBP3 protein serum levels in SGA children compared for length with AGA children.

1:400), p73 antibody Ab-2 (ER-15 clone; Oncogene, San Diego, CA; 1:200), antibody anti–cyclin A (Santa Cruz Biotechnology; 1:200), antibody anti–cyclin D1 (Santa Cruz Biotechnology; 1:200), antibody anti–cyclin E (Santa Cruz Biotechnology; 1:200), anti-actin Ab-1 antibodies kit (Calbiochem, San Diego, CA; 1:2000), and IGFBP3 antibody (Santa Cruz Biotechnology; 1:600).

Bound primary antibodies were visualized using Lumi-Light Western Blotting Substrate (Roche, Milan, Italy) on a UVITEC Cambridge Camera.

RNA extraction from cell lines. Total cellular RNA was extracted from HCT116, HCT116p53$^{-/-}$, MCF-7, and 293 T-Rex (CAT, p53wt, p73α) cells using the RNeasy mini kit (Qiagen, Venlo, Netherlands). Purified RNA was then quantified using the NanoDrop 1000 Spectrophotometer (Thermo Scientific, Waltham, MA), and RNA quality was determined by running aliquots on the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA).

RNA extraction from blood. Peripheral blood samples were collected in PAXgene Blood RNA Tubes (BD, Hombrechtikon, Switzerland; cat. no. 762162). The sample was gently inverted and stored at –80°C within 2 h of collection. RNA was purified using the manual procedure according to the PAXgene Blood RNA Kit Handbook (BD, cat. no. 762174). Freshly extracted RNA was dosed, and RNA integrity was additionally assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies).

	SGA children (n = 16)	AGA children (n = 14)	p Value
Male/female	10/6	11/3	—
Gestational age (wk)	38.61 ± 1.33	39.03 ± 0.98	ns
Current age (yr)	9.08 ± 3.87	9.53 ± 3.62	ns
Birth weight (g)	2360.18 ± 410.92	3350.12 ± 0.37	<0.05
Birth weight SD	–1.98 ± 0.91	0.01 ± 0.84	<0.05
Birth length (cm)	45.55 ± 2.13	50.15 ± 1.14	<0.05
Birth length SD	–1.88 ± 0.89	–0.03 ± 0.70	<0.05
Height (cm)	123.00 ± 24.1	140.02 ± 13.80	<0.05
Height SD	–1.85 ± 1.02	0.69 ± 0.90	<0.05
IGF-1 (ng/ml)	268.53 ± 183.7	201.4 ± 63.78	ns
IGF-1 SD	0.0 ± 1	0.45 ± 1.46	ns
IGFBP3 (ng/ml)	2899.28 ± 975.26	3864.71 ± 397.32	<0.05
IGFBP3 SD	0.0 ± 1	0.0 ± 1	ns

Data are presented as mean and SD. ns = not significant.

TABLE 1: Clinical and biochemical characteristics of the study population.

RT-qPCR analysis

Reverse transcription of 200–500 ng of total RNA was performed using QuantiTect Reverse Transcription kit (Qiagen), according to the manufacturer's instruction. RT-qPCR experiments were performed in duplicate on the ABI PRISM 7900HT platform (Applied Biosystems, Life Technologies, Carlsbad, CA), using 1 µl cDNA as the template for each reaction with TaqMan Universal PCR Master Mix (Applied Biosystems, Life Technologies). TaqMan assays from Applied Biosystems were used for the amplification of IGFBP3 (Hs00181211_m1), p53 (Hs00153349_m1), p63 (Hs00978340), p73 (Hs01056230_m1), GADPH (Hs99999905_m1), and HPRT1 (Hs03929098_m1) transcripts. Results were first analyzed in SDS 2.2.1 software and then exported into Microsoft Excel to be further analyzed by using geNorm, which output GAPDH and HPRT1 as best housekeeping genes for cell culture and blood samples, respectively. The reported data represent the average of at least three independent experiments and are shown with their SEs. Two-tailed Student's *t* tests were performed to assess the statistical significance of gene expression level differences observed between the SGA and AGA children. In this study, a p value < 0.05 was considered to be statistically significant.

Cell cycle analysis. Asynchronous, starved, and refed HCT116 cells, transfected with the empty pRS (control) or four different p73 shRNAs (Origene), were used for this assay. The total cell population, including floating and adherent cells, was harvested at the given time point; washed twice with 1× phosphate-buffered saline; and treated with 150 mg/ml RNase A, 5 mg/ml propidium iodide (PI), and NP-40 0.1% at room temperature for 1 h. The cells were analyzed in a FACScalibur; cell cycle and apoptosis analyses were performed using ModFit analysis software (Becton Dickinson, Franklin Lakes, NJ).

Transfections and luciferase assays. The fragments containing the p53-REs of the human *IGFBP3* gene (boxes A, B, and C) were amplified from the human genomic DNA and cloned in the pGL-3 basic plasmid (Promega, Madison, WI). Human H1299 cells (1×10^5) were cultured 24 h before transfection (60–80% confluency). Transient reporter assays were performed as described in *Transfections*. Each well was cotransfected using TransIT-LT1 Transfection Reagent (Mirus Bio, Madison, WI) according to the manufacturer's instructions, with either empty pcDNA3 vector or containing p53wt, p73α, TAp63α, ΔNp63α, or their mutated forms p53R175H, p73αV156A, TAp63αR279Q, and ΔNp63αR279Q (150 ng); the recombinant reporter vector containing the p53-REs of the human *IGFBP3* gene (box A, box B, and box C; 1 µg); and the *Renilla* pRL-SV40 vector (Promega; 10 ng). Thirty-six hours after transfection, H1299 cells were lysed in passive lysis buffer (Promega), and the luciferase assay was performed using the Dual Luciferase assay system (Promega) according to the manufacturer's instructions. Data were normalized to the *Renilla* reporter signal. The results reported represent the average of at least three independent experiments and are shown with the SDs.

Chromatin immunoprecipitation assay. T-Rex-293-CAT, T-Rex-293-p53wt, and T-Rex-293-p73 cells were cultured in 15-cm culture dishes for 24 h after the induction of the different proteins. Proteins were cross-linked to DNA in living nuclei, and a chromatin immunoprecipitation assay was performed as previously described (Tullo *et al*., 2003). Five micrograms of the following antibodies was used to immunoprecipitate the DNA–protein complexes: p53 antibody DO-1 (Santa Cruz Biotechnology), p63 antibody H137 (Santa Cruz Biotechnology), p73 antibodies H-79 and C-20 (Santa Cruz Biotechnology), acetylated H4-histone antibody (Upstate, Lake Placid, NY), or unrelated control anti-Flag antibody (Sigma-Aldrich, St. Louis, MO). DNA fragments were analyzed by PCR using specific primers.

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