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Liposarcoma initiated by FUS/TLS-CHOP: the FUS/TLS domain plays a critical role in the pathogenesis of liposarcoma

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The most common chromosomal translocation in liposarcomas, t(12;16)(q13;p11), creates the FUS/TLS-CHOP fusion gene. We previously developed a mouse model of liposarcoma by expressing FUS-CHOP in murine mesenchymal stem cells. In order to understand how FUS-CHOP can initiate liposarcoma, we have now generated transgenic mice expressing altered forms of the FUS-CHOP protein. Transgenic mice expressing high levels of CHOP, which lacks the FUS domain, do not develop any tumor despite its tumorigenicity *in vitro* and widespread activity of the EF1α promoter. These animals consistently show the accumulation of a glycoprotein material within the terminally differentiated adipocytes, a characteristic figure of liposarcomas associated with FUS-CHOP. On the contrary, transgenic mice expressing the altered form of FUS-CHOP created by the in frame fusion of the FUS domain to the carboxy end of CHOP (CHOP-FUS) developed liposarcomas. No tumors of other tissues were found in these transgenic mice despite widespread activity of the EF1α promoter. The characteristics of the liposarcomas arising in the CHOP-FUS mice were very similar to those previously observed in our FUS-CHOP transgenic mice indicating that the FUS domain is required not only for transformation but also influences the phenotype of the tumor cells. These results provide evidence that the FUS domain of FUS-CHOP plays a specific and critical role in the pathogenesis of liposarcoma. *Oncogene* (2000) **19,** 6015–6022.

Keywords: chromosomal abnormality; malignant solid tumors; adipose tissue; cancer developement

Introduction

Liposarcoma is the most common soft tissue malignancy in adults accounting for at least 20% of all sarcomas in this age group (Mack, 1995). Multiple histologic subtypes of liposarcoma are recognized including well differentiated, dedifferentiated, myxoid, round cell, and pleomorphic. The histologic group is predictive of both the clinical course of the disease and the ultimate prognosis (Chang *et al*., 1989). The myxoid/round cell liposarcomas exhibited the characteristic t(12;16)(q13;p11) translocation (Knight *et al*., 1995). The molecular characterization of this translocation revealed a fusion between the *CHOP* gene and a gene called *TLS/FUS* (Crozat *et al*., 1993; Rabbitts *et al*., 1993), resulting in the RNA-binding domain of TLS/FUS being replaced by the basic leucine zipper domain of CHOP, which confers the ability to form protein dimers (Ron and Habener, 1992; Crozat *et al*., 1993). The *TLS/FUS* gene has also been shown to fuse with the gene for Ets-like protein ERG as a result of the translocation t(16;21)(p11;q22) in some patients with acute nonlymphoblastic leukemia (Shimizu *et al*., 1993; Ichikawa *et al*., 1994; Panagopoulos *et al*., 1994, 1995). The portion of TLS/FUS that is present in the TLS/FUS-CHOP and FUS-ERG fusion proteins is similar and this part has been shown to be an autonomous transcriptional activation domain (Prasad *et al*., 1994; Sánchez-García and Rabbits, 1994; Zinszner *et al*., 1994). In the FUS-CHOP fusion, transcriptional activation is therefore specifically conferred on the chimeric protein by the *FUS* segment after the translocation event (Sánchez-García and Rabbitts, 1994). *In vitro* the transforming effects of *FUS-CHOP* have been demonstrated in fibroblasts (Pérez-Losada *et al*., 2000), but curiously not in 3T3-L1 cells under conditions expected to yield oncogenic effects (Zinszner *et al*., 1994), suggesting that the function of FUS-CHOP is influenced by the cell environment *in vitro*. In transgenic mice in which FUS-CHOP is ubiquitously expressed, we have shown that the overexpression of FUS-CHOP results in most of the symptoms of human liposarcomas, identifying the FUS-CHOP as the main mechanism in human liposarcoma. Whereas interference with the normal process of differentiation may contribute to the oncogenic potential of FUS-CHOP fusion protein, it is a property that they share with the nontransforming germline CHOP gene product (Zinszner *et al*., 1994; Batchvarova *et al*., 1995). CHOP expression is tightly regulated. Under normal conditions the gene is repressed, and CHOP mRNA and protein are absent from cells (Fornace *et al*., 1989; Price and Calderwood, 1992; Carlson *et al*., 1993; Wang *et al*., 1996). In contrast with CHOP, FUS is a constitutively activated gene (Aman *et al*., 1996). These observations support a model whereby the FUS-CHOP fusion gene represents a gain-of-function mutation of CHOP that deregulate both gene expression and protein activity. *In vitro* studies have demonstrated that injection of CHOP-expressing cells into nude mice produced only small

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tumors with longer latency than that of FUS-CHOP expressing cells, suggesting that the presence of amino-terminal sequence from FUS in the chimeric protein is necessary for realizing its full oncogenic potential (Zinszner *et al*., 1994).

In order to examine *in vivo* how FUS-CHOP can initiate liposarcoma, we have now generated transgenic mice expressing two altered forms of the FUS-CHOP protein. Transgenic mice expressing high levels of CHOP, which lacks the FUS domain, do not develop any tumor despite its tumorigenicity *in vitro* and widespread activity of the EF1α promoter. These animals consistently show the accumulation of a glycoprotein material within the terminally differentiated adipocytes, a characteristic figure of liposarcomas associated with FUS-CHOP. On the contrary, transgenic mice expressing the altered form of FUS-CHOP created by the in frame fusion of the FUS domain to the carboxy end of CHOP (CHOP-FUS) specifically developed liposarcomas. The characteristics of the liposarcomas arising in the CHOP-FUS mice were very similar to those previously observed in our FUS-CHOP transgenic mice indicating that the function of FUS-CHOP is independent from the chimeric junction domain and demonstrating that the FUS component of the fusion protein acts as an autonomous domain which is required not only for transformation but also influences the phenotype of the tumor cells. These results provide evidence that the FUS domain of FUS-CHOP plays a specific and critical role in the pathogenesis of liposarcoma.

Results

Generation of transgenic mice expressing altered forms of FUS-CHOP

We previously developed a mouse model of liposarcoma by expressing FUS-CHOP from the elongation factor 1α (EF-1α) promoter in murine mesenchymal stem cells (Pérez-Losada *et al*., 2000). In order to understand *in vivo* how FUS-CHOP can initiate liposarcoma, we have now generated transgenic mice expressing two altered forms of the FUS-CHOP protein. The first truncated protein (CHOP) lacks the FUS domain (Figure 1a). *In vitro* this truncated protein, unable to activate transcription (Prasad *et al*., 1994; Sánchez-García and Rabbitts, 1994; Zinszner *et al*., 1994), is able to interfere with the normal process of adipocyte differentiation (Zinszner *et al*., 1994), a property which shares with FUS-CHOP. However, CHOP induces growth arrest (Barone *et al*., 1994), but CHOP-expressing cells into nude mice produced tumors with longer latency than that of FUS-CHOP-expressing cells (Zinszner *et al*., 1994).

The second altered form of FUS-CHOP was created by the in frame fusion of the FUS domain to the carboxy end of CHOP (CHOP-FUS) (Figure 1a). The reason to make this second altered form is to investigate the contribution of both the FUS domain and chimeric junction of FUS-CHOP to the genesis of liposarcoma *in vivo*. This artificial CHOP-FUS chimeric protein cannot be recognized by specific antibodies raised against both the amino and the carboxy ends of the wild-type FUS-CHOP protein, indicating that the junction generates different domains in CHOP-FUS and FUS-CHOP proteins. As FUS contributes a strong transcriptional activation domain to the fusion protein (Prasad *et al*., 1994; Sánchez-García and Rabbitts, 1994; Zinszner *et al*., 1994), before producing transgenic animals we validated the characteristics of our CHOP-FUS cDNA construct on transcriptional activity. The effects of CHOP-FUS were similar to FUS-CHOP (data not shown), indicating that the FUS domain is an autonomous domain which does not require the junction to the amino end of CHOP at least for transcriptional activity.

In order to examine the direct consequences of *CHOP* and *CHOP-FUS* expression *in vivo* we follow the same strategy than we previously used for FUS-CHOP (Pérez-Losada *et al*., 2000). Therefore, *CHOP* and *CHOP-FUS* cDNAs were cloned downstream the EF-1α promoter (EF-CHOP and EF-CHIOP-FUS) to direct expression to all tissues (Figure 1b) and injected into C57BL/6 $\times$ CBA fertilized eggs. Three founders were obtained for EF-CHOP (IS345, IS329, and IS267) and two founders for EF-CHOP-FUS (IS418, and IS438) (Table 1). All founder lines for both EF-CHOP and EF-CHOP-FUS showed germline transmission of the transgene. Transgene expression was observed in all lines and tissue expression of the EF-CHOP and EF-CHOP-FUS transgenes were demonstrated by Western blot analysis with an 9E10 monoclonal antibody (Figure 1c). The elongation factor 1α (EF-1α) promoter successfully drove the expression of *FUS-CHOP* in different tissues in the mouse (Figure 1c), showing no preferential expression in fat-tissues and transgenic expression was detected in both male and female mice. Normal mouse fat cells treated in parallel did not exhibit any expression.

EF-CHOP transgenic mice develop normally

Cohorts of transgenic mice were generated to analyse the effect of the *CHOP* gene (Table 1). CHOP transgenic mice developed healthy, were fertile and none of the CHOP transgenic mice developed liposarcomas in up to 24 months of observation (Table 1). This result contrasts with our observations in the FUS-CHOP transgenic mice. In these mice, liposarcomas developed in both independently derived founder lines beginning at 3 months of age, and by 10 months of age liposarcoma had appeared in all mice of the two highest expressing FUS-CHOP lines (Pérez-Losada *et al*., 2000). Although liposarcomas did not develop in the CHOP transgenic mice, we investigated whether the CHOP protein altered white adipose tissue (WAT) development in these mice. The histopathological analyses of the white adipose depots in CHOP transgenic mice did not evidence any pathological change within the terminally differentiated adipocytes (Figure 2). On the contrary, CHOP transgenic mice had a normal architecture of the tissue and we did not observe any shift in the WAT toward immature in the CHOP transgenic mice (Figure 2). The histopathological examination only showed the accumulation of an unknown material in the mature adipocytes. This material was not present in normal WAT neither in normal BAT (Figure 2). As FUS-CHOP is associated with myxoid liposarcomas, we used a routine cytochemical technique to provide clues as to composition.

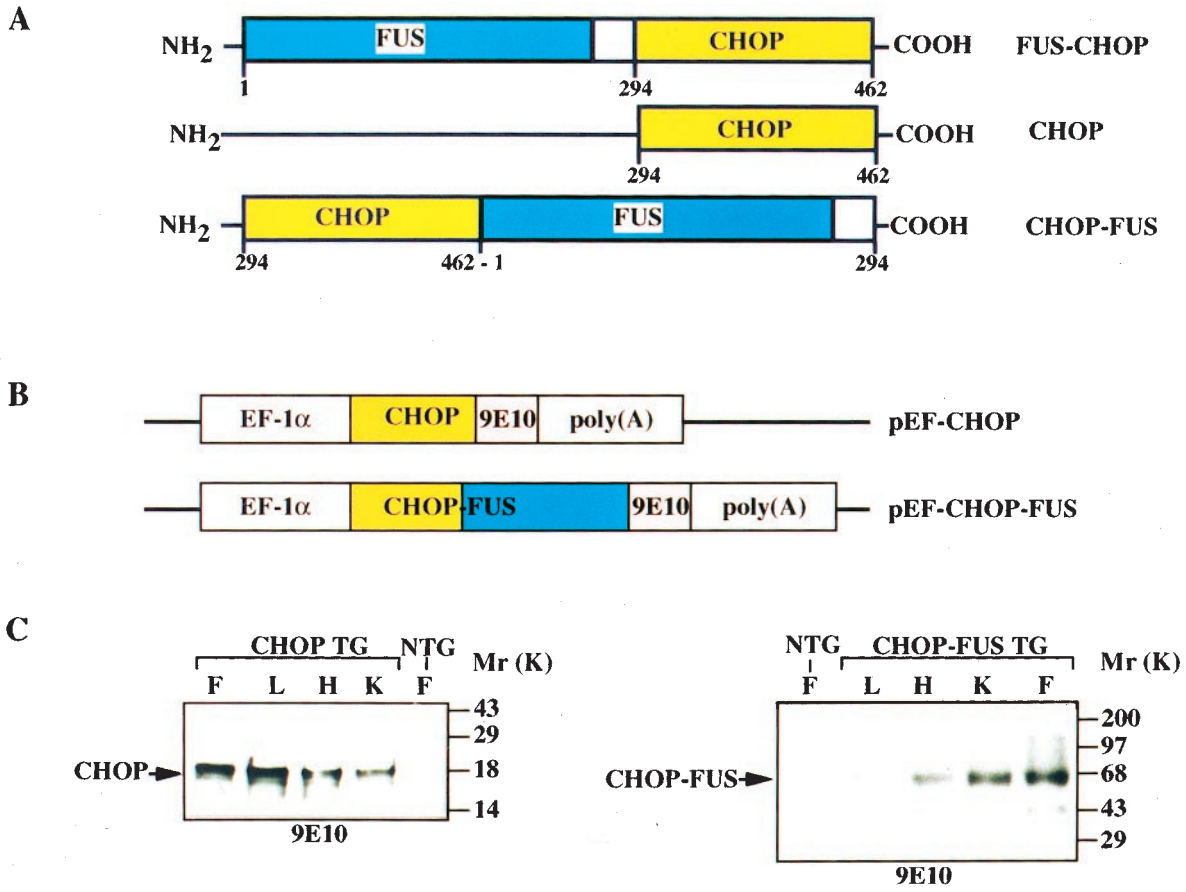

Figure 1 EF-CHOP and CHOP-FUS transgenic mice: transgene construct and expression. (**a**) Schematic representation of the FUS-CHOP protein created as a result of the t(12;16)(q13;p11) chromosomal translocation and the altered forms of FUS-CHOP, CHOP and CHOP-FUS proteins, used in this study. (**b**) The human CHOP and CHOP-FUS cDNAs containing a tag-sequence for the human c-Myc (amino acids 408–439) at the 3′ end were cloned into the pEF-BOS vector, which contains the EF-1 promoter sequences and polyadenylation and splice signals from the human G-CSF gene, to give pEF-CHOP and pEF-CHOP-FUS. Expression of CHOP and CHOP-FUS proteins in transgenic mice was determined by Western blot analysis. (**c**) Expression of the transgene was demonstrated in the liver (L), heart (H), kidney (K), and fat (F) of a 2-month-old EF-CHOP (line IS345) and EF-CHOP-FUS (line IS438) transgenic (TG), respectively. As a negative control we used the fat from a 2-month-old nontransgenic (NTG) littermate. The location of CHOP and CHOP-FUS is as indicated

Table 1 Incidence and age of tumor-onset in the transgenic mice expressing altered forms of FUS-CHOP protein

Transgenic line	*Mice autopsied**	*Mice with tumor (%)•*	*Age in months at tumor onset*	*Tumor type*
IS345 (EF-CHOP)	125	0	-	-
IS 329 (EF-CHOP)	92	0	-	-
IS267 (EF-CHOP)	103	0	-	-
IS438 (EF-CHOPFUS)	54	54 (100%)	5–9	Liposarcoma
IS418 (EF-CHOPFUS)	79	79 (100%)	5–8	Liposarcoma

*Number of EF-CHOP mice analysed during their lifespan and EF-CHOPFUS mice studied during or after the period of liposarcoma development. •Number of EF-CHOPFUS mice that had liposarcoma as determined at autopsy and percentage of tumor incidence

The Periodic Acid-Schiff (PAS) is a specific stain for all cells containing glycogen. This cytochemical technique clearly demonstrates the presence of PAS-positive material within CHOP-adipocytes (Figure 3f–h). Positive reaction is also present in myxoid liposarcomas arising in mice expressing FUS-CHOP (Figure 3e). Control cells derived from adipose tissues coming from wild-type mice are PAS-negative (Figure 3a–d). Although the promoter drove the expression of CHOP in different tissues in the mice (Figure 1c), the examination of other organs in these CHOP transgenic mice did not evidence other abnormalities.

The inability of CHOP to generate tumors and perturb WAT development *in vivo* contrasts with its effects *in vitro* where CHOP shares some properties of FUS-CHOP protein: the capacity to inhibit adipocyte differentiation and generate tumors in nude mice (Zinszner *et al*., 1994). These capacities contrast with the ability of CHOP to induce growth arrest (Barone *et al*., 1994) and enhance differentiation of erythroid cells (Cui *et al*., 2000). Thus, our results demonstrate that some of the *in vitro* properties of CHOP do not correlate *in vivo* with the normal development of CHOP mice and indicate that the properties conferred

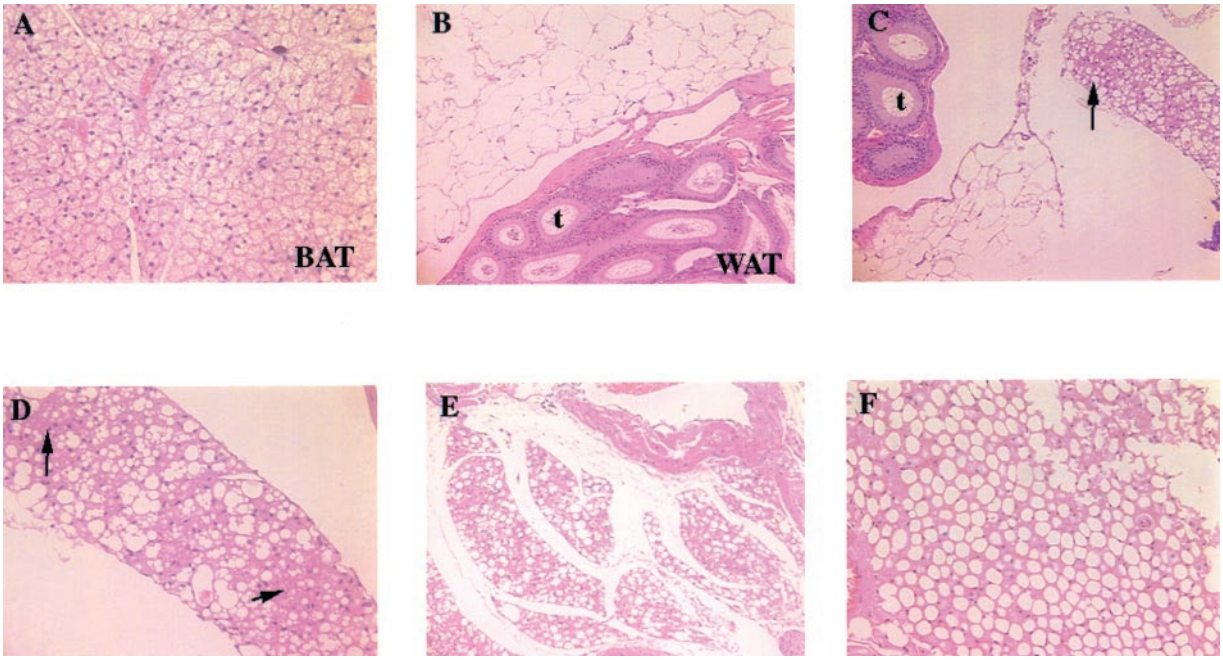

Figure 2 Histologic appearance of the adipose tissues in EF-CHOP transgenic mice. The adipose tissue in EF-CHOP transgenic mice in the region of the testis (**c**,**d**) or intestine (**e**,**f**) shows the accumulation of an unknown material within adipocytes compared to control brown adipose tissue (**a**) and normal white adipose tissue (**b**). The original magnification is $\times 10$ for (**a**,**b**,**c**,**e**) and $\times 20$ for (**d**,**f**). BAT, brown adipose tissue; WAT, white adipose tissue; t, testis

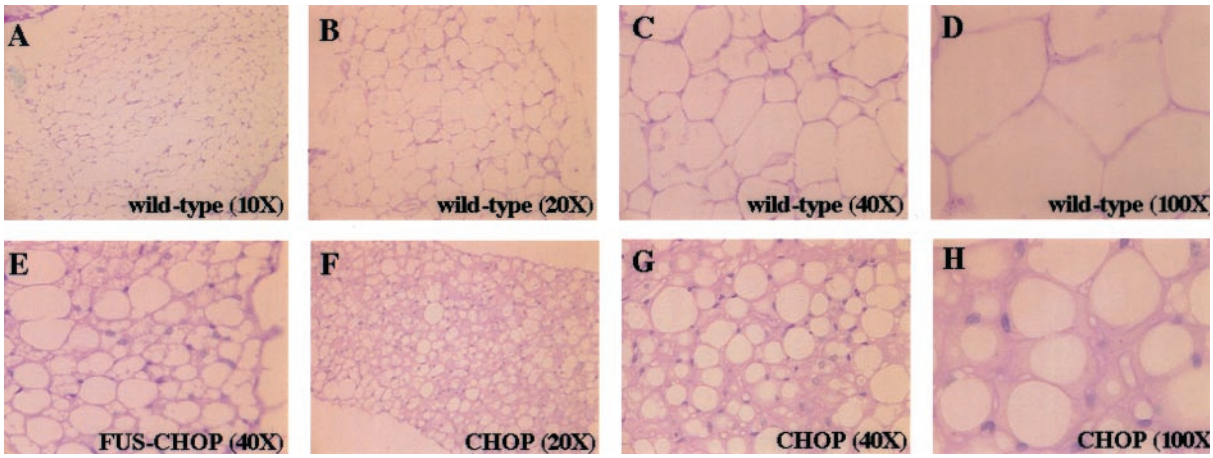

Figure 3 Histologic appearance of the adipose tissue in EC-CHOP transgenic mice stained with the PAS technique. The PAS is a specific stain for all cells containing glycogen. This cytochemical technique clearly demonstrates the presence of PAS-positive material within CHOP-adipocytes (**f**,**g**,**h**). Positive reaction is also present in myxoid liposarcomas arising in mice expressing FUS-CHOP (**e**). Control cells derived from adipose tissues coming from wild-type mice are PAS-negative (**a**–**d**)

by FUS to the FUS-CHOP fusion protein are essential for the genesis of liposarcoma.

CHOP-FUS transgenic mice develop liposarcomas

We next investigated the contribution of the FUS domain to the genesis of liposarcoma *in vivo* by the generation of cohorts of transgenic mice to analyse the effect of the *CHOP-FUS* fusion gene (Table 1). Uniformly, male and female EF-CHOP-FUS transgenic mice showed the same symptoms beginning as early as 8 weeks of age with increasing signs over time leading to death in 100% of the mice by 12 months of age (Table 1). The survival was similar in the two founder lines. At the time of autopsy, these animals had developed palpable masses involving the adipose tissues, which, upon dissection, revealed white adipose pads 4–100-fold normal size). Tumor infiltration of non-adipose tissues was not usually visible and was confirmed microscopically. All these animals had visible masses as early as 8–10 weeks. In addition, some animals not included in the Table 1 never developed palpable masses but showed microscopic tumors upon dissection when they were sacrificed at 10 weeks of age. This examination is consistent with adipose tissue disease. However, no tumors of other tissues were found in these EF-CHOP-SUS transgenic mice, despite widespread activity of the EF-1α promoter.

We next defined the adipose tissue disease generated in our EF-CHOP-FUS transgenic mice. Detailed

analysis of the tumor cells in the EF-CHOP-FUS transgenic mice established the diagnosis as malignant liposarcomas. Haematoxilin/eosin staining showed that the tumor cells had an adipoblast morphology (Figure 4). Histologically, all tumors were diffuse liposarcomas composed of medium to large cells with round nuclei and were very similar to human liposarcomas (see http://www-medlib.med.utah.edu/WebPath/NEOHTML/NEOPLO52.html) and liposarcomas generated in FUS-CHOP transgenic mice (Pérez-Losada *et al*., 2000). The histopathological analyses of the white adipose depots in both EF-CHOP-FUS transgenic lines revealed multiple abnormalities. Some contained only modest morphological changes consisting primarily of an increase in nuclear size and number per microscopic field, but the white adipose tissue (WAT) of the majority of the mice had a much more hypercellular appearance and contained scattered cells with enlarged pleomorphic nuclei. Similarly to FUS-CHOP transgenic mice, CHOP-FUS animals with large sarcomas had relatively small WAT depots. In these white fat pads the normal architecture of the tissues was largely or completely disrupted by the extensive proliferation of white adipocytes. Virtually all of the white adipocytes in these dysplastic depots had some degree of cytological atypia such as cellular or nuclear enlargement and pleomorphism (Figure 4). Nests of compact anaplastic liposarcoma cells were often scattered throughout these depots. Similarly to FUS-CHOP transgenic mice, WAT deposits in these animals were generally affected at least with abnormal adipocytes and, in some cases, foci of sarcomas were clearly discernible. Pathological changes were not observed in the brown adipose tissue (BAT) of these transgenic mice. Histopathological examination of the other organs of these animals showed no other abnormalities, with the exception of skeletal abnormalities. These skeletal defects include scoliosis and curvature and lesions extended over the entire spine, however, the larger lesions were predominantly present in the thoracic and upper lumbar regions, which were similar to FUS-CHOP transgenic mice (Pérez-Losada *et al*., 2000). We did not find a focus of tumor cells in any of these organs. The tumors were invariably found in the WAT depots, usually multifocally in any WAT depot.

To test the malignant nature of cells from the EF-CHOP-FUS transgenic mice, 1×10^6 cells from liposarcoma tumors were injected subcutaneously into 40 day-old nude mice. Thirty-two mice injected developed progressively growing tumors within 4–7 weeks of transplantation. The transplanted cells developed into the same kind of tumor as defined by histological analysis. Furthermore, the origin of liposarcoma cells from donor mice was confirmed by PCR analysis revealing the presence of the *CHOP-FUS* gene product. These data define the tumors generated in the EF-CHOP-FUS transgenic mice as malignant liposarcomas. We next examined the expression of adipocyte-specific genes in order to define the level at

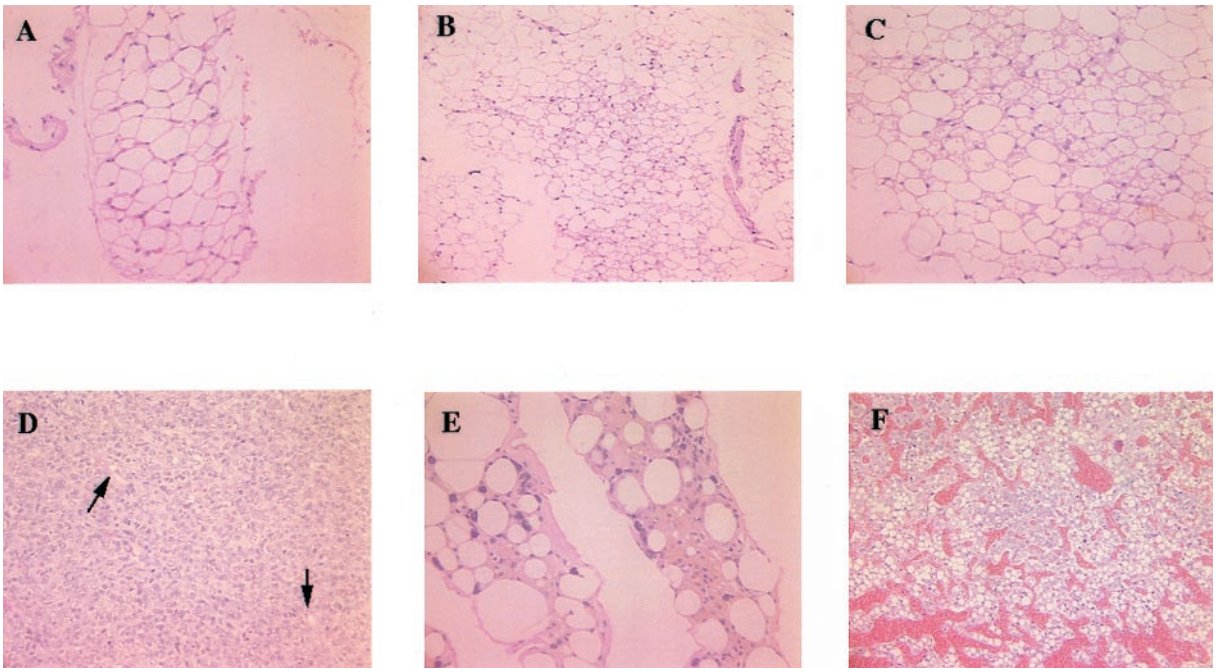

Figure 4 Liposarcoma development and WAT abnormalities in EF-CHOP-FUS transgenic mice. Hematoxylin-eosin stained section of the adipose tissue from an EF-CHOP-FUS transgenic mouse that developed a liposarcoma in the region of the testis shows the effacement of the normal fat architecture (**b**) compared to control mice (**a**). At high magnification (**c**), the presence of the lipoblasts are visible in this liposarcoma which correspond to a high grade myxoid/round cell type. (**d**) Microscopic appearance of a liposarcoma arising in the inter-escapular region. Note the lipid droplets in some, but not all, cells as well as the nuclear pleomorphism. The arrow indicates the presence of vacuolated, lipid filled cells, to determine the cell of origin. This liposarcoma gave metastasis to different regions. The histology confirmed the tumor origin as shown in (**e**). (**f**) Retroperitoneum was another primary site for liposarcomas in the EF-CHOP-FUS transgenic mice. Although the liposarcoma disease in EF-CHOP-FUS transgenic mice was very similar, the level of adipoblast infiltration in the two different EF-CHOP-FUS transgenic lines revealed by histological analysis was not identical. Similar results were found in multiple sections from EF-CHOP-FUS transgenic and nontransgenic mice. The original magnification is $\times 10$ for (**a**,**b**,**d**,**e**,**f**); $\times 20$ for (**c**)

which the differentiation is blocked in CHOP-FUS liposarcomas. Similarly to FUS-CHOP transgenic mice (Pérez-Losada *et al*., 2000) and despite their block in differentiation each liposarcoma examined was found to express significant levels of PPAR-γ RNA, c/EBPβ, δ and α and aP2 comparable to that of normal fat and FUS-CHOP liposarcomas (data not shown). These results suggest that liposarcomas in CHOP-FUS transgenic mice have been transformed at a point in the differentiation process after induction of PPARγ expression. Moreover, PPARγ is not expressed in any other type of soft tissue sarcoma (Tontonoz *et al*., 1997) and can be considered a sensitive marker for distinguishing liposarcoma from other histologic types of soft tissue sarcoma. Therefore, EF-CHOP-FUS transgenic mice reproduce the same phenotype with which the *FUS-CHOP* fusion gene is associated both in human pathology and transgenic mice (Pérez-Losada *et al*., 2000). These observations demonstrate that the chimeric junction sequence is not necessary for the genesis of liposarcoma and two independent domains within the FUS-CHOP protein are the main mechanisms by which FUS-CHOP generates liposarcoma.

Embryonic fibroblasts from CHOP mice differentiate into adipocytes but adipocyte differentiation is blocked in embryonic fibroblasts from CHOP-FUS mice

To investigate whether CHOP-FUS protein retains the same adipocyte inhibition capacity than FUS-CHOP and the difference in the results obtained from the present analysis of CHOP transgenic mice and previous *in vitro* culture systems where CHOP interferes with the normal process of adipocytic differentiation (Zinszner *et al*., 1994; Kuroda *et al*., 1997; Adelmant *et al*., 1998), we prepared primary embryonic fibroblasts from CHOP and CHOP-FUS mice and examined their differentiation properties as previously described (Pérez-Losada *et al*., 2000). The embryonic fibroblasts from each line were cultured to confluent and then treated with the standard differentiation induction medium. As shown in Figure 5, embryonic fibroblasts from wild-type and CHOP mice differentiated into adipocytes and accumulated many lipid droplets in response to hormonal stimulants. In contrast, CHOP-FUS embryonic fibroblasts can hardly differentiate into adipogenic cells and accumulate very few lipid droplets, similarly to FUS-CHOP embryonic fibroblasts (Figure 5). The Oil-Red-O staining demonstrated clear differences in adipocyte phenotype (Figure 5) and, similarly to FUS-CHOP MEFs, MEFs from CHOP-FUS transgenic mice only expressed the earliest markers of differentiation (data not shown), which corresponds to preadipocytes. These results show that adipocyte differentiation was blocked in cultured CHOP-FUS embryonic fibroblasts but not in MEFs derived from CHOP transgenic mice treated with adipogenic hormones. Interference with the normal process of differentiation contributes to the oncogenic potential of FUS-CHOP fusion protein (Pérez-Losada *et al*., 2000) and this capacity is retained by CHOP-FUS. On the contrary, CHOP is not able to interfere with this normal process of adipocytic differentiation, explaining why CHOP mice do not develop liposarcomas.

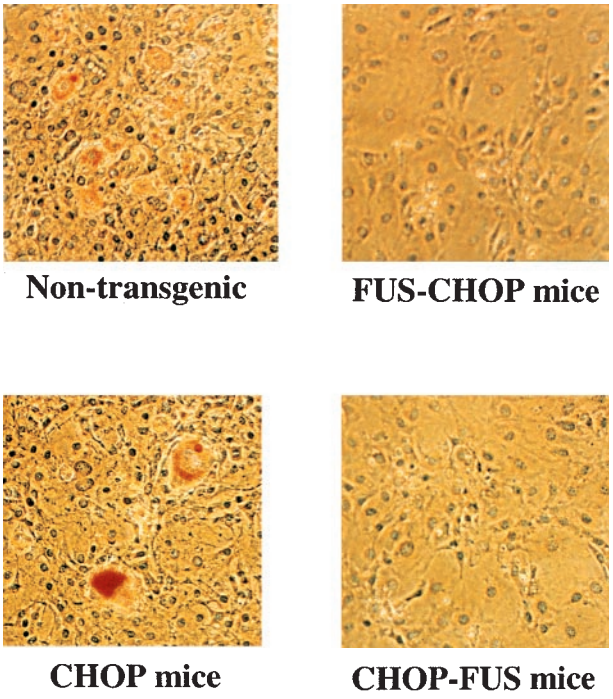

Figure 5 Adipogenic differentiation from primary embryonic fibroblasts expressing altered forms of FUS-CHOP protein. Primary embryonic fibroblasts from each transgenic line were cultured in the presence of standard differentiation induction medium (containing 0.5 mM isobutylmethylxantine, 1 μM dexamethasone, 5 μg/ml insulin and 10% FBS). After 8 days of differentiation, cells were observed by light microscopy with Oil-Red-O staining. Cells from nontransgenic, FUS-CHOP, CHOP and CHOP-FUS mice are shown. The original magnification is $\times 20$. This experiment was repeated three times using cells prepared from all transgenic lines and from different embryos and similar results were obtained

Concluding remarks

We have demonstrated that directing expression of the FUS-CHOP fusion protein to immature mouse cells initiated sarcomas with adipose features (Pérez-Losada *et al*., 2000). We have now similarly expressed altered forms of FUS-CHOP. The results show that the truncated protein CHOP which lacks the FUS activation domain does not generate any tumors neither affect the development of WAT *in vivo*. Concordant with these data, adipocyte differentiation was not blocked in cultured CHOP embryonic fibroblasts treated with adipogenic hormones. Thus, these results show that the transcriptional activation by FUS is indispensable for tumorigenesis. However, the genesis of liposarcoma could be explained by properties of FUS-CHOP protein which derived from the junction sequence. For this purpose we fused the FUS domain to the carboxy end of CHOP. The consequent overexpression of CHOP-FUS resulted in most of the symptoms of human liposarcomas, including the presence of lipoblasts with round nuclei, accumulation of intracellular lipid, induction of adipocyte-specific genes and a concordant block in the differentiation program. No tumors of other tissues were found in these transgenic mice despite widespread activity of the EF1α promoter, underscoring the relevance of the relationship between FUS-CHOP and the cell environment.

In both EF-CHOP-FUS transgenic lines multiple palpable adipose tumors synchronously arise. Their simultaneous occurrence, extensively involving all adipose areas, is consistent with the diffuse, polyclonal origin of these tumors, similarly to FUS-CHOP transgenic mice. These data and our previous observations (Pérez-Losada *et al*., 2000) strongly support that *FUS-CHOP* is a single-step action oncogene which transforms the target cell. In fact, if one considers the combined effects of FUS-CHOP and CHOP-FUS on differentiation and proliferation controls, it seems reasonable to think that the single chromosome abnormality that leads to the generation of this protein may be all that is needed for the liposarcoma target cells to become transformed (Sánchez-García, 1997). The fact that liposarcomas only arise in CHOP-FUS transgenic but not in CHOP transgenic mice establishes the FUS domain as a key determinant of human liposarcomas. Although, *in vitro* it is possible that FUS-CHOP and its normal counterpart CHOP inhibit differentiation by interfering with the activation of transcription by C/EBP family members. *In vivo*, however, it seems that CHOP is not able to sequester C/EBPs. Alternatively, FUS-CHOP may modify the C/EBP response by altering the subset of DNA-binding sites through which C/EBPs activate transcription. Further studies to identify the target genes and proteins that are regulated by FUS-CHOP using our transgenic mice will produce a clearer picture of the development defect in liposarcoma. Our results demonstrate that the order of the two sequences (FUS and CHOP) is interchangeable, but they do not clarify whether the relationship between the FUS and CHOP domains is *in cis* or *in trans*. Further studies will be required to clarify this point.

Materials and methods

The generation and screening of transgenic mice

PCR amplification of the CHOP portion of FUS-CHOP was done to facilitate cloning by adding restriction enzymes using primers that hybridize to the 5′ and 3′ ends of the CHOP cDNA. The artificial CHOP-FUS in frame fusion was created by PCR (fusion point: 5′...(chop)....CTGCACCAAGCA-CTCGAGATGGCCTCAAAC...(FUS)....3′). The products were cloned into the M13mp19 vector, added to 3′ end an in-frame sequence coding for a MYC-tag recognized for the 9E10 antibody, and sequenced. The cDNA was cloned into a plasmid, pEF-BOS, containing sequences of the EF1-α promoter, followed by a polylinker region linked to the poly(A) adenylation signal from human G-CSF cDNA as described (Mizushima and Nagata, 1990). Linear DNA fragments for microinjection were obtained by *Hind*III digestion and injected by the INIA Transgenic Facility into CBA $\times$ C57BL/6J fertilized eggs. Transgenic founders were identified by Southern-blot analysis through detection of the novel EF-CHOP and EF-CHOP-FUS constructs from samples of DNA extracted from tails, respectively.

Histological analysis

CHOP-FUS and CHOP transgenic mice were subjected to standard necropsy. All major organs were closely examined under the dissecting microscope, and samples of each organ were processed into paraffin, sectioned and examined histologically. Tumor specimens from the CHOP-FUS transgenic mice were fixed into 10% formalin overnight, then processed, embedded in paraffin, and 6 μm sections were stained with hematoxylin and eosin and photographed. All tissue samples were taken from homogenous and viable portions of the resected sample by the pathologist and fixed within 2–5 min of excision. Hematoxylin- and eosin-stained sections of each soft tissue sarcoma were reviewed by a single pathologist (T Flores) and classified according to histologic type and grade. Histological classification was based solely on morphologic pattern recognition using conventional diagnostic criteria. Adipose tissues from CHOP transgenic mice were also stained with the Periodic Acid-Schiff (PAS) reaction.

Cell transplantation to nude mice

Liposarcoma tumors were dispersed in Dulbecco's modified Eagle's medium containing 5% fetal calf serum by mechanical shearing onto a large mesh grid and injected subcutaneously into 40 day-old nude mice. Approximately 10^6 cells were injected at each site. Mice were monitored once a week and were killed for histopathologic studies and collection of tissues for DNA analyses when moribund.

Preparation of primary embryonic fibroblasts and induction of adipogenic differentiation

Primary embryonic fibroblasts were harvested from 14.5 d.p.c. embryos. Cells were cultured at 37°C in Dulbeccos-modified Eagle's medium (DMEM; Boehringer Ingelheim) supplemented with 10% heat-inactivated FBS (GIBCO/BRL). Cells were plated to 24-well or 60 mm plastic dishes and propagated to confluence. Two days later, medium was replaced with standard differentiation induction medium containing 0.5 mM isobutylmethylxantine (Sigma), 1 μM dexamethasone (Sigma), 5 μg/ml insulin (Sigma) and 10% FBS (GIBCO/BRL). This medium was removed every other day. After 8 days, the appearance of cytoplasmic lipid accumulation was observed by microscopy with Oil-Red-O staining. The Oil-Red-O staining was performed as follows: cells were washed with phosphate-buffered saline (PBS), and then stained with 60% filtered Oil-Red-O stock solution (0.15 g of Oil-Red-O (Sigma) in 50 ml of isopropanol) for 30 min at 37°C. Cells were washed first with 60% isopropanol and then briefly with water and visualized.

Western blot analysis

Single cell suspensions coming from different tissue samples were analysed by immunoblotting procedures as essentially as described (Castellanos *et al*., 1997). Lysates were run on a 10% SDS–PAGE gel and transferred to a PVDF membrane. After blocking, the membrane was probed with c-Myc (9E10) mouse monoclonal antibody (Santa Cruz), which is specific for human c-Myc (amino acids 408–439). Reactive bands were detected with an ECL system (Amersham).

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