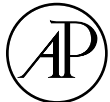

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Biochemical and Biophysical Research Communications 302 (2003) 114–120

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E2F1 blocks and c-Myc accelerates hepatic ploidy in transgenic mouse models

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Received 20 January 2003

Abstract

Previously, we have shown that over-expression of either E2F1 or c-Myc promotes hepatocarcinogenesis and that E2F1 mice acquire HCC more rapidly than c-Myc transgenic mice. We also found that co-expression of E2F1/c-Myc further accelerates liver cancer development. Here we describe that the deregulated expression of these two transcription factors also affects hepatic ploidy during post-natal liver growth and before the onset of tumors. Oncogenic activity of E2F1 and/or c-Myc was associated with a persistent increase in hepatocyte proliferation. However, E2F1-mediated cell proliferation favored the predominance of diploid cells characteristic of pre-neoplastic type of liver growth whereas c-Myc functioned to accelerate age-related hepatocyte polyploidization. Similarly, proliferative advantage conferred by co-expression of E2F1 and c-Myc increased the frequency of diploid cells at a young age. Thus, the opposing effects of E2F1 and c-Myc on hepatocyte ploidy suggest that these two transcription factors have different mechanisms by which they control liver proliferation/maturation and ultimately, carcinogenesis.
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Keywords: Ploidy; E2F1; c-myc; Liver

The E2F1 and c-Myc transcription factors are both involved in regulating key cellular activities including growth and death [1–3]. When over-expressed, E2F1 and c-Myc are capable of driving quiescent cells into S phase in the absence of other mitogenic stimuli [4,5]. In addition to providing a continuous proliferative signal, E2F1 and c-Myc are also potent inducers of apoptosis and operate at least through one common pathway involving p53 [6–8]. Also, deregulated expression of c-Myc and E2F1 is frequently found in cancer cells [3,9–13]. To dissect the cellular and molecular pathways regulated by these two transcription factors and their role in carcinogenesis, we have developed several transgenic models in which over-expression of E2F1 and/or c-Myc was targeted to the liver. We found that although both c-Myc and E2F1 mono-transgenic mice were prone to liver cancer, E2F1 mice developed HCC more rapidly and with a higher frequency [14]. Moreover, the combined expression of these two transcription factors dramatically accelerated HCC growth compared to either E2F1 or c-Myc mono-transgenic mice.

All three transgenic lines showed a low but persistent elevation of hepatocyte proliferation before an onset of tumor growth. In the liver, cell proliferation is unique in that it is the result of distinct, yet coupled, processes: cell multiplication, cell polyploidization, and cytoplasmic hypertrophy. An alternation between mitotic and polyploidizing cell cycles is a common feature of normal post-natal liver growth [15]. This is in contrast to preneoplastic growth which is dominated by a mono-nucleated diploid cell population [16,17]. It has been postulated that the switch from growth by polyploidization to growth by cell multiplication increases the number of diploid cells thus providing an advantage for expansion of initiated cells [16,18,19]. Because the outcome of cell proliferation depends on the nature of inductive signals and varies considerably upon application of different growth stimuli

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0006-291X/03/$ - see front matter © 2003 Elsevier Science (USA). All rights reserved.
doi:10.1016/S0006-291X(03)00125-6

[20–23], we took advantage of our transgenic models to examine whether over-expression of c-Myc and/or E2F1 can affect liver ploidy. The results demonstrate that c-Myc and E2F1, two critical regulators of liver growth, have opposing effects on hepatocyte ploidy and suggest distinct differences by which these two transcription factors control liver proliferation/maturation.

Materials and methods

Construction of fusion genes and generation of transgenic mice. The Alb/c-*myc* (c-Myc) and Alb/E2F1 (E2F1) transgenic mice were generated by using mouse c-*myc* and human E2F1 recombinant cDNAs, respectively, driven by the albumin promoter as described previously [24] in C57Bl/6J $\times$ CBA/J background. Double transgenic mice (ME) were generated by crossing homozygous E2F1 (line [8]) with homozygous c-Myc [166.8] mice. Animal study protocols were conducted according to the National Institutes of Health guidelines for animal care.

Determination of DNA content. DNA was isolated from weighed liver specimens utilizing a simplified mammalian DNA isolation procedure described by Laird et al. [25]. Total DNA content was then determined by multiplying the mg of DNA per gram of tissue by the liver weight in grams.

Determination of nuclear ploidy. Hepatocytes were isolated by the two-step collagenase perfusion of the liver followed by isodensity centrifugation in Percoll as described previously [26]. Nuclei were isolated from 1×10^6 cells and stained with propidium iodine (PI) utilizing the Cycle Test Plus DNA Reagent Kit (Becton–Dickinson, San Jose, CA). Nuclear DNA content was then measured using CellQuest Software in a FACScan Flow cytometer (Becton–Dickinson).

Results

E2F1 and c-Myc do not cooperate to enhance cell proliferation

Previously we found that in the c-Myc single-transgenic mouse a continuous high cell proliferation preceded the appearance of pre-neoplastic lesions [27]. This was also true, although to a lesser extent, in the E2F1 mouse [14]. In the present work, we quantified the proliferative activities in E2F1, c-Myc/E2F1, and c-Myc transgenic livers (Fig. 1) at 15 weeks of age. At this time point, all of the transgenic mouse lines had a high incidence (>60%) of hepatic dysplasia (Calvisi and Conner, unpublished results). The mitotic indices were equivalent in c-Myc/E2F1, and c-Myc livers, $0.135 \pm 0.026\%$ and $0.140 \pm 0.05\%$, respectively, and were 2-fold higher than the mitotic index in E2F1 ($0.06 \pm 0.01\%$). The mitotic activity in Wt mice was exceedingly low at $0.025 \pm 0.03\%$.

c-Myc over-expression facilitates development of polyploidy at the preneoplastic stage

In rodents livers, different growth stimuli may cause diverse effects on ploidy and nuclearity [21,28,29]. To clarify the ploidy-specific alterations as related to hepatocarcinogenesis, we next sought to determine whether mitogenic stimuli provided by c-Myc and/or E2F1 transgene contribute to hepatocyte polyploidization or selective multiplication of a specific ploidy class. Since mitotic polyploidization occurs predominantly from 2 to 3 weeks post-natally and increases with age [15], we performed a FACS analysis of the DNA content in hepatocyte nuclei isolated from 4- to 15-week-old mice. In young Wt livers, the majority of nuclei had a diploid DNA content with a smaller proportion of tetraploid nuclei (Figs. 2A and 3A). As mice aged, the number of

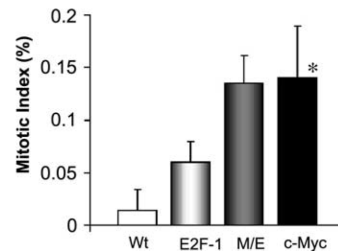

Fig. 1. Proliferation indices in Wt and preneoplastic liver tissue from E2F1, c-Myc/E2F1, and c-Myc mice at 15 weeks of age. Indices were scored on H&E-stained liver sections as described in Materials and methods. Each bar represents the means $\pm$ SEM of at least five mice per group. $P < 0.05$ when compared with Wt value by Student's t test.

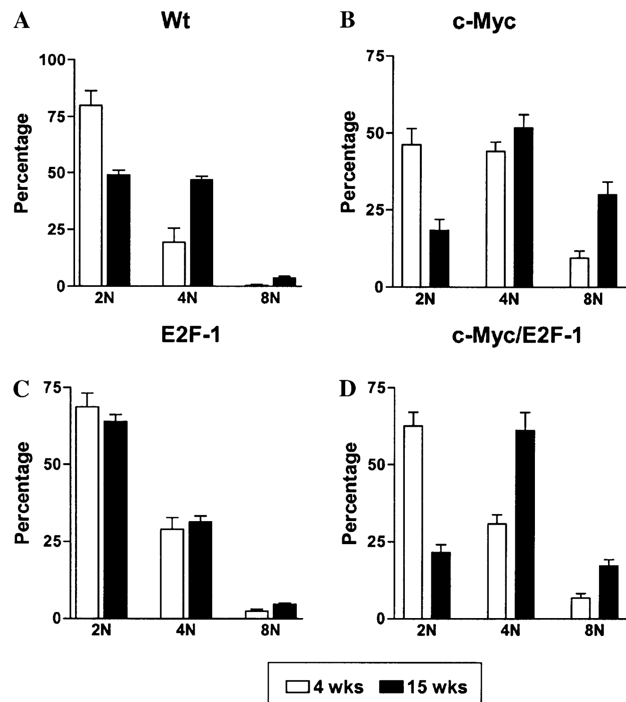

Fig. 2. Analysis of nuclear ploidy during preneoplastic stage of hepatocarcinogenesis. Mice at 4 and 15 weeks of age from each group were subjected to determination of nuclear ploidy. Hepatocytes were isolated as described in Materials and methods and purified nuclei were stained with PI and analyzed using flow cytometry. Each bar represents the means $\pm$ SEM of 3–5 separate experiments.

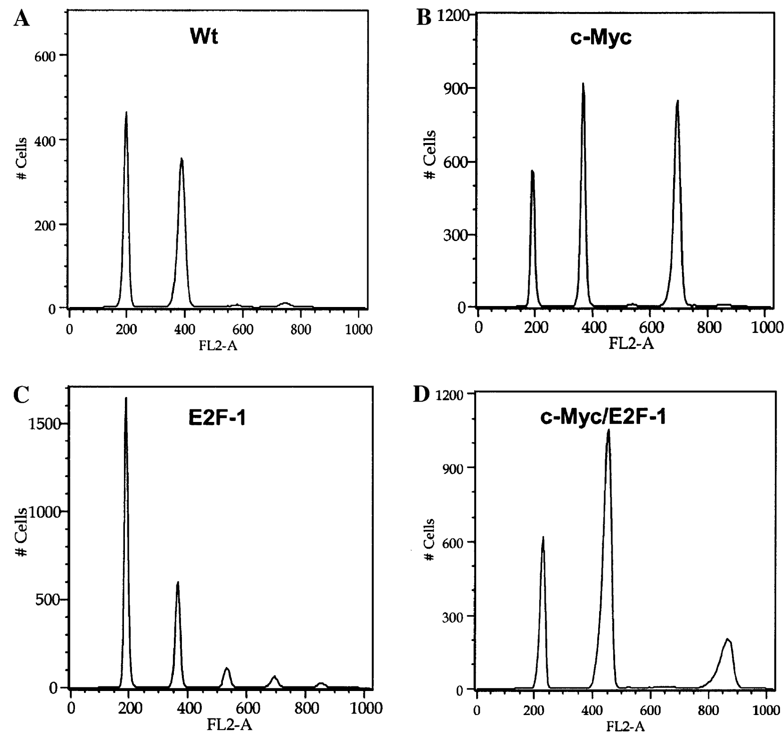

Fig. 3. Ploidy distributions of isolated nuclei from 15 week livers from Wt, E2F1, c-Myc/E2F1,and c-Myc transgenic mice. Hepatocytes were isolated as described in Materials and methods and purified nuclei were stained with PI and analyzed using flow cytometry. Representative wild-type, E2F1, c-Myc,and c-Myc/E2F1 histograms are shown.

tetra- and octaploid nuclei increased consistent with the previous findings of others [15,18,30].

In comparison, c-Myc mice demonstrated a premature polyploidization (Fig. 2B). Thus, at 4 weeks of age the number of 2N nuclei in c-Myc livers was almost 2-fold less ($46.2 \pm 5.2\%$ vs. $83.6 \pm 6.3\%$ in Wt) while the proportion of 4N nuclei increased more than 2.5-fold ($44.0 \pm 3.0\%$ vs. $16.3 \pm 6.2\%$ in Wt). The most prominent ploidy alteration was the increase in the fraction of hepatocytes with octaploid nuclei. In young c-Myc mice, the frequency of octaploid nuclei was about 200-fold higher than that found in age-matched Wt controls (10% vs. 0.05%). The percentage of polyploid cells continued to rise in 15-week-old c-Myc livers with the majority of hepatocytes having nuclei with 4N and 8N DNA content (Figs. 2B and 3B). Accordingly, there was an increase in the number of binucleated hepatocytes ($72.2 \pm 8.4\%$ vs. $66.5 \pm 6.0\%$ in Wt livers), as well as an increase in the average cell size (data not shown). These data show that polyploid hepatocytes arise earlier in c-Myc livers and their number increases faster than in Wt livers, suggesting that c-Myc accelerates the spontaneous development of polyploidy.

E2F1 over-expression blocks age-related polyploidization during post-natal liver ontogenesis

In striking contrast, E2F1 hepatocytes did not undergo normal polyploidization with aging (Figs. 2C and 3C). In 4-week-old E2F1 mice, similar to Wt controls, cells with diploid nuclei comprised the most common ploidy class although the relative frequencies of tetra- and octaploid nuclei were higher, resulting in a slight increase in the mean ploidy levels (2.7N vs. 2.3N in Wt mice) (Fig. 2C). However, differently from both Wt and c-Myc mice, the majority of E2F1 nuclei remained in diploid state. The ploidy distribution profiles were almost identical in E2F1 mice at 4 and 15 weeks of age (Fig. 2C). In addition, the percentage of binucleated hepatocytes was 23–30% lower in E2F1 livers than in Wt, c-Myc, or c-Myc/E2F1 livers. The ratios of mononucleated to binucleated in Wt, E2F1, c-Myc, and c-Myc/E2F1 cells were 0.50, 0.72, 0.39, and 0.48, respectively. These results indicate an increased proportion of mononucleated cells in E2F1 livers and a predominance of binucleated cells in c-Myc livers. Together, the findings suggest that E2F1 over-expression interferes with a normal process of mitotic polyploidization in the liver facilitating multiplication of diploid hepatocytes rather than their polyploidization.

In c-Myc/E2F1 mice, the age-related changes in ploidy distribution resembled those found in both c-Myc and in E2F1 single transgenic mice. At a young age, c-Myc/E2F1 mice, similar to E2F1 mice, retained significantly more diploid nuclei than c-Myc mice, $62.5 \pm 4.5\%$ and $68.7 \pm 4.5\%$ vs. $46.2 \pm 5.2\%$, respectively (Figs. 2D and 3D). When FACS analysis was performed with whole cells, this difference increased up

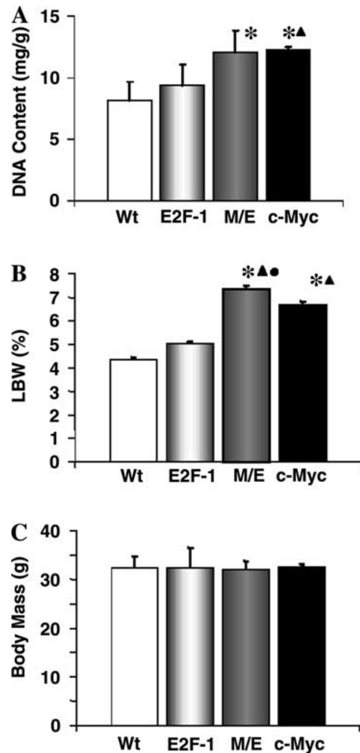

Fig. 4. Parameters of liver growth in wild-type and transgenic mice at 15 weeks of age. (A) Total DNA content (mg) determined by multiplying the mg of DNA per gram of tissue by the liver weight in grams. (B) Liver weight expressed as the ratio of liver weight to body weight. (C) Body mass in grams (g). Each bar represents the means ± SEM of five separate experiments. $P < 0.001$ when compared with Wt (*), E2F1 (▲), and (●) c-Myc by Student's *t* test.

to 5-fold, indicating that the diploid profile of young E2F1 and c-Myc/E2F1 cells was due to predominance of mono-nucleated diploid hepatocytes (data not shown). However, as mice aged, the majority of c-Myc/E2F1 hepatocytes, similar to c-Myc cells but in contrast to findings in E2F1 cells, became polyploid (Figs. 2D and 3D). Consistent with a more progressive polyploidization, the DNA content was significantly higher in both c-Myc/E2F1 and c-Myc livers, 12.0 ± 0.98 and 12.3 ± 0.23 mg/g liver weight, respectively, as compared to 8.2 ± 0.7 and 9.4 ± 0.89 mg/g for Wt and E2F1 (Fig. 4A). Of note, the livers from 15-week-old c-Myc/E2F1 mice retained a slightly higher proportion of diploid nuclei, $21.6 \pm 2.5\%$ as compared to $18.4 \pm 3.5\%$ in c-Myc mice (Figs. 2B and D). Since the livers from the double transgenic mice were larger than those of the c-Myc mice (Figs. 4B and C) the difference in the absolute number of 2N nuclei would be even more pronounced (Fig. 2D).

Discussion

In an effort to dissect whether the proliferative advantage conferred by c-Myc and/or E2F1 over-expression affects the predominance of diploid or polyploid cells, we compared the ploidy profiles in single and double transgenic mice during early post-natal liver growth. We find that deregulated expression of *myc* oncogene accelerated the spontaneous development of polyploidy in the liver of transgenic mice. In striking contrast to the effect of c-Myc, E2F1 transgene blocked age-related increase in ploidy levels causing accumulation of diploid hepatocytes and thus enlarging the population of susceptible cells at the preneoplastic stages of liver cancer development. Other known modulators of ploidy in the liver are the tumor suppressor p53, pRb, and the cell cycle inhibitor p21 [31–35]. In addition, genes involved in control of the cell cycle progression, such as cyclin A, cyclin B, cyclin D3, and cyclin E, have been described to affect the ploidy status in other cell types [36–40].

In the liver, polyploidization is a normal and essential component of organ growth. In newborn mice and rats, the majority of hepatocytes are diploid mononucleated cells that divide mitotically increasing the number of hepatocytes. After weaning, when metabolic load on liver increases, hepatocytes progress through a modified or polyploidizing cell cycle which contains gaps and S phases but proceeds without cytokinesis [41]. This results in the formation of the first polyploid cell, bi-nucleated with diploid nuclei, thus increasing the cell ploidy but not cell number. The subsequent proliferation of bi-nucleated hepatocytes occurs with a fusion of mitotic nuclei during metaphase giving rise to mono-nucleated cells with higher levels of ploidy. During normal liver ontogenesis, a polyploidizing cell cycle without cytokinesis alternates with a mitotic cycle of bi-nucleated cells, resulting in progressive and irreversible increases either in cell or nuclear ploidy [41]. The cell ploidy is most extensive in a mouse liver, but also common for rat and human livers [42,43]. In humans, the average levels of cell and nuclear ploidy are relatively low; however the pattern of hepatocyte polyploidization is similar. At maturity and especially during aging, the rate of hepatocyte polyploidization increases, and in the elderly individuals binucleated and polyploid hepatocytes constitute about one-half of liver parenchyma [44].

In contrast to the post-natal liver growth, a switch from the normal process of progressive polyploidization to a diploid growth pattern seems to be a general feature of liver carcinogenesis [16,45]. The block of age-related polyploidization, as observed in the E2F1 expressing livers, would seem to indicate that multiplication of diploid cells provides a selective growth advantage at the early stage of hepatocarcinogenesis [43,46]. Since a mutagenic insult upon a diploid genome is more likely to result in a malignant transformation than a similar insult towards a polyploid genome, diploid cells would be a primary target

population for tumor development [43]. The early development of focal lesions composed of small cells, a prominent feature of E2F1 and c-Myc/E2F1 associated hepatocarcinogenesis, was complemented by a higher frequency of diploid nuclei, which may reflect the early "switch" to preneoplastic growth in these mice. In sharp contrast to both E2F1 and Wt livers, c-Myc dysplastic livers exhibited much more advanced polyploidization, in agreement with our previous findings [26].

The comparison of ploidy profiles in our transgenic models provides evidence that over-expression of c-Myc and/or E2F1, two critical components of cell cycle control, can modulate hepatocyte ploidy by promoting either the polyploidizing cell cycle which increases the fraction of polyploid cells or mitotic cell cycle shifting the ploidy profile towards 2N cells. These findings are consistent with numerous other observations. Over-expression of E2F1 blocked polyploidization of transgenic megakaryocytes suggesting that E2F1 interferes with terminal differentiation [47]. On the other hand, over-expression of c-Myc has been found to increase cell ploidy in a variety of experimental systems [48,49]. Given the fact that c-Myc is a very potent inducer of DNA synthesis, it is thought that its ability to override the checkpoint that normally prevents the replication of damaged DNA results in the generation of polyploid cells [50–52]. Excess of c-Myc activity was also sufficient to abolish a spindle assembly checkpoint and induce tetraploidy as has been shown in experiments with inhibitors of microtubule assembly or cleavage furrow formation [53].

The significance of accelerated polyploidization in c-Myc transgenic livers is not immediately obvious and requires further investigation. The ability of c-Myc to drive the proliferation of damaged cells may be critical to its oncogenic function. There is growing evidence that an increase in ploidy can compromise the proliferative and functional capacity of a cell [54]. Accordingly, acceleration of polyploidization in c-Myc transgenic livers was associated with a steady decline in mitotic activity consistent with a reduced growth potential of polyploid cells relative to diploid cells [26,55–57]. c-Myc mice also experienced a persistent liver injury as evidenced by a significant elevation of circulating levels of aspartate aminotransferase, alanine aminotransferase, and alkaline phosphatase [26,55]. Consequently, hepatic progenitor cells were activated as reflected by the appearance of a frequent oval/ductular proliferation. Similar changes in ploidy were also found in the c-Myc/E2F1 double transgenic mice, which exhibited not only more severe dysplasia but an early increase in focal lesions, resulting in a relative abundance of diploid cell population as compared to c-Myc mice. These data suggest that co-expression of both E2F1 and c-Myc contributes to an early establishment of preneoplastic diploid cell population.

Our results show that polyploidization, a carefully controlled process, is distorted in liver by the over-expression of E2F1 and c-Myc. The close association between ploidy disruption and neoplastic development in the liver suggests that characterization of mechanism(s) of ploidy changes may provide important insight into the molecular pathways of HCC formation.

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