

Suppression of the Tumorigenicity of Mutant p53-transformed Rat Embryo Fibroblasts through Expression of a Newly Cloned Rat Nonmuscle Myosin Heavy Chain-B

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Abstract

In our previous study, a rat homologue of human nonmuscle myosin heavy chain-B (nmMHC-B) was identified by mRNA differential display comparing of transformed against nontransformed Rat 6 cells overexpressing mutant p53^{val135} gene. The nmMHC-B was found to be expressed in normal Rat 6 embryo fibroblast cell line, but markedly suppressed in the mutant p53^{val135}-transformed Rat 6 cells. To examine the possible involvement of nmMHC-B in cell transformation, we first cloned and sequenced the full length cDNA of rat nmMHC-B, which was then cloned into an ecdysone-expression vector. The resulting construct was introduced into the T2 cell line, a mutant p53^{val135}-transformed Rat 6 cells lacking the expression of the endogenous nmMHC-B. The clonal transfectants, expressing muristerone A-induced nmMHC-B, displayed a slightly flatter morphology and reached to a lower saturation density compared to the parental transformed cells. Reconstitution of actin filamental bundles was also clearly seen in cells overexpressing the nmMHC-B. In soft agar assays, nmMHC-B transfectants formed fewer and substantially smaller colonies than the parental cells in response to muristerone A induction. Moreover, it was strikingly effective in suppressing the tumorigenicity of the T2 cells when tested in nude mice. Thus, the nmMHC-B, known as a component of the cytoskeletal network, may act as a tumor suppressor gene. Our current finding may reveal a novel role of nmMHC-B in regulating cell growth and cell signaling in nonmuscle cells.

Introduction

Myosin, a cytoskeletal protein conserved among all eukaryotic cells, plays a major role in muscle contraction. The conventional myosin (class II myosin) molecule possesses a hexameric structure composed of one pair of heavy chains of approximately 200 kD and two pairs of light chains of about 15-28 kD. Myosin is expressed in both muscle and non-muscle cells. The role of myosin in muscle contraction is well known, whereas the function of myosin in nonmuscle cells is less clear. Evidence suggests that myosin plays a role in cytokinesis (De Lozanne and Spudich, 1987), cell proliferation (Rovner *et al.*, 1986; Simons *et al.*, 1992), secretion (Ludowyke *et al.*, 1989) and migration (Warrick and Spudich, 1987). Defects in myosins are thought to be associated with several genetics lesions (for review see Gulick and Rayment, 1997). Two major isoforms of nonmuscle myosin heavy chain A and B (nmMHC-A and -B) have been identified in vertebrate cells. The cDNAs of nmMHC have been cloned from human, mouse, chicken, and xenopus (Choi *et al.*, 1996; Phillips *et al.*, 1995; Saez *et al.*, 1990; Katsuragawa *et al.*, 1989; Bhatia-Dey *et al.*, 1993). A recent study indicates that nmMHC-B is required for normal development of mouse heart and that its absence results in abnormal development of cardiac myocyte (Tullio *et al.*, 1997). Unique forms of isoform B have also been identified in chicken and human neuronal cells (Takahashi *et al.*, 1992; Itoh and Adelstein, 1995) and was reportedly involved in neuronal differentiation. A study by Mummert and Schengrund (1997) indicated that nmMHC-B has a function in neurite formation.

In our recent effort to elucidate the role of the mutant p53 gene in neoplastic transformation, a temperature sensitive mutant p53^{val135} (valine at position 135) was introduced into normal Rat 6 embryo fibroblasts that retained

the endogenous wild type p53. One of the resulting p53^{val135}-overexpressors, R6#13-8, showed no obvious alteration in growth properties; however, they did exhibit frequent spontaneous transformation in prolonged cultures. The T2 clonal cell line, derived from one of the spontaneous transformed foci of R6#13-8 cultures, showed typical transforming morphology and formed tumors in nude mice. In addition, the cells no longer expressed the vectored p53, suggesting secondary changes occurred in these cells leading to cell transformation (Yam *et al.*, 1999). In an effort to identify events related to mutant p53^{val135}-induced malignant phenotypes, R6#13-8, and its derived spontaneous transformant T2 cell line were employed for mRNA differential display. After a systematic screening with 80 sets of primers, three significant cDNA fragments (Yam *et al.*, 1999) were identified. One of these was a 351-bp fragment found down-regulated in T2 and it shared 93% homology with the 3' terminus of the human nmMHC-B gene. Using the 351 bp fragment as a probe in a Northern blot analysis, a 7.6 kb transcript corresponding to the reported human nmMHC-B was detected among the normal Rat 6, untransformed Rat 6 derivatives overexpressing p53^{val135}, but not in the spontaneous transformants derived from R6#13-8 cultures. The levels of transcript seem to be inversely correlated with the degree of transformation of the tested cell lines.

Although nmMHC-B is involved in diverse cellular functions, there is no indication that it participates in cell transformation. Early studies on cytoskeletal proteins such as actin, tropomyosin and vinculin have provided evidence that reduction of cytoskeletal proteins are often a key feature of cell transformation (for a review, see Ben-Ze'ev, 1997). Later studies also showed that overexpression of α -actinin, vinculin or tropomyosin could reverse transforming

phenotypes of certain transformed cells (Glück *et al.*, 1993; Fernández *et al.* 1992, Boyd *et al.*, 1995). Moreover, an interesting notion has emerged that some cell adhesion molecules, known to be intimately associated with cytoskeletal network, may work as tumor suppressors (Tsukita *et al.*, 1993). All these observations prompt us to hypothesize that suppression of nmMHC-B in T2 cells might contribute in part to the malignant phenotypes of T2 cells, which may be reversed by re-introducing the gene back to the cells.

To investigate the role of nmMHC-B in cell transformation, T2 transformed cells that expressed negligent levels of nmMHC-B were transfected with the rat nmMHC-B (designated as r-nmMHC-B) gene under an ecdysone expression system. Results showed that overexpression of r-nmMHC-B slightly flattened cell morphology, lowered saturation densities and partially reversed the anchorage-independent growth of T2 cells. Intriguedly, r-nmMHC-B transfected T2 cells were non-tumorigenic when inoculated into nude mice. Based on this observation, r-nmMHC-B may act as a tumor suppressor gene at least in cells transformed by the mutated p53 tumor suppressor gene.

Results

Suppression of nmMHC-B, but not -A isoform was observed in spontaneous transformant T2 cell line.

In organisms and cell types studied so far, the conventional myosin II consists of two major isoforms, MHC-A and -B, encoded by different genes (Simons *et al.*, 1991). Using A- and B- specific polyclonal antibodies as probes, the extracts of Rat 6, R6#13-8, and T2 cells were analyzed by immunoblotting (Fig. 1A). The nmMHC-A and -B both yield proteins with 220 kD in size. In order to verify the

specificity of each antibody, Cos7 cells (known to express only B isoform), and human serum platelets (known to express only A isoform) were included in the study as positive controls. Interestingly, the expression of A-isoform is unaffected in the T2 cell line. Similar results were obtained by immunofluorescent staining (Fig. 1B). Although both isoforms are coexpressed in most cell types, lacking expression of one or the other isoform has no apparent effect on cell viability as evidenced by the growth of T2, Cos7 cells and others, such as the rat mast cell line RBL-2H3 described by Choi *et al.* (1996).

Cloning of the full-length rat nmMHC-B cDNA.

To confirm and further characterize the putative gene homologous to nmMHC-B, the 351 bp rat cDNA fragment previously identified was used as a probe to screen a rat brain cDNA library (Stratagene #936501). Two isolated overlapping positive clones provide sequence information for a 7648 bp full length cDNA of r-nmMHC-B (GenBank AF139055), which consists of an open reading frame of 5928 bp coding region, preceded by 188 nucleotides and tailed with 1532 bp of untranslated region. Compared the homologues of human and chicken (GenBank M69181) (Phillips *et al.*, 1995) & (GenBank M93676) (Katsuragawa *et al.*, 1989), the rat homologue has a longer 5' untranslated region (UTR), which might be derived from a minor upstream transcription initiation site, as suggested by Weir and Chen (1996). The 3' UTR is 69% homology to the human sequences (Phillips *et al.*, 1995), however, there are two stretches of sequences (6226-6707 & 6941-7620) which share 86% and 78% to those of human, suggesting that the well conserved 3' UTR may play an important role in regulating nmMHC-B function. The r-nmMHC-B exhibit 99 and 96% homology in amino acid sequences, and 89

and 83% homology in nucleotide sequences, to human and chicken respectively (Phillips *et al.*, 1995; Katsuragawa *et al.*, 1989). The deduced amino acid sequences of r-nmMHC-B were compared to those from human and chicken shown in Fig. 2. The reported unique sequence insertions (Takahashi *et al.*, 1992; Itoh *et al.*, 1995) are not found in r-nmMHC-B.

Construction and expression of r-nmMHC-B vector in T2 cells.

To address whether the suppression of nmMHC-B in T2 cells might have contributed to its acquired transforming phenotypes, the full length r-nmMHC-B cDNA was cloned into pIND/Hygro vector, which contains the ecdysone response element sequences and the hygromycin gene. The resulting vector pIND/nmMHC-B together with the vector pVgRXR carrying both the retinoid X receptor and an ecdysone receptor and a zeocin gene were cotransfected into T2 cells, followed by zeocin and hygromycin selections. Two stable transfectants, T2/MHC-B#33 (MHC#33) and T2/MHC-B#44 (MHC#44), which showed inducible nmMHC-B expression upon induction in a dosage dependent manner (Fig. 3A) were selected for later study. A T2 derivative-T2C expressing only the pIND and pVgRXR backbone vectors was also established as a control cell line. Based on the screenings on nmMHC-B expression, all the tested transfectants showed basal levels of nmMHC-B in the absence of the inducer, indicating that there is a leakage in the system. In general, MHC#44 displays lower background level of nmMHC-B under uninduced condition and is more responsive upon muristerone A induction compared to the MHC#33 clone (Fig. 3A). Neither the parental T2 nor T2C expresses any detectable nmMHC-B protein.

With regard to morphology, the parental T2 and vectors-expressing T2C cells showed a typical transforming morphology with short, refractile cell bodies that were densely packed, while the stable MHC-B transfectants displayed a slightly flattened morphology in the absence of inducer (Fig. 3B). MHC#44 was flatter than MHC#33. Addition of muristerone A seemed to further flatten the morphology of both clones slightly, while imposed no effect on T2 or T2C cells (Fig. 3B).

Immunofluorescent localization of nmMHC-B in the transfectants.

We then further characterized MHC#44 by immunofluorescent staining and observed that the subcellular distribution of the exogenous r-nmMHC-B was quite different from that observed in Rat 6 or R6#13-8 cells (Fig. 3C). In Rat 6 cells, the endogenous nmMHC-B was diffusely throughout the cytoplasm, whereas the exogenous r-nmMHC-B was densely accumulated around the nuclear envelope of MHC#44, irrespective of stages of cell growth or division, which became more prominent upon addition of the inducer. As expected, nmMHC-B is not detectable in T2 cells, induced or uninduced (Fig. 3C).

The nmMHC-B-expressing cells showed reduction in saturation density and anchorage-independent growth.

The effect of nmMHC-B on growth rate and saturation density was examined in MHC#44 clone. Figure 4A shows growth curves of MHC#44 cells in the absence or presence of 2, 5, & 10 μ M of muristerone A. There are no apparent differences in growth rate among the experimental groups; however, the saturation density was steadily decreased as the concentrations of inducer steadily increased. Early

western blotting showed that the induced level of r-nmMHC-B was proportionally correlated with the dosage of the applied inducer. In the same experiment, a single “day 8” time point of T2 cells in the absence or presence of 10 μ M inducer was included. The saturation density of MHC#44 reached only 46% and 32 % of the control T2 cells in both uninduced and induced conditions. To ensure that the growth retardation observed in MHC#44 cells was not simply due to the nonspecific toxic effect of muristerone A, T2 and MHC#44 were treated with various dosages of muristerone A for 8 days, and then were counted for cell number. Compared to the untreated T2 cells, the treated T2 show 15 and 17% growth reduction, whereas MHC#44 cells show 50 and 58% reduction under the treatment of 5 and 10 μ M muristerone A, respectively (Fig. 4A). Despite the fact that muristerone A was slightly toxic at high concentration, the growth inhibition seen in MHC#44 cells was much more striking than in the T2 cells. We therefore conclude that reduced saturation density was mainly due to the effect of r-nmMHC-B expressed in MHC#44 cells. It is worth mentioning that there was no obvious cell death in the induced cultures, suggesting that overexpression of r-nmMHC-B might have restored the contact inhibition property of the T2 cells, but did not exert a direct killing effect on cells. In fact, the r-nmMHC-B expressing cells appeared healthy, although somewhat slow growing, under high dosages of muristerone A.

To investigate the effect of nmMHC-B on the malignant phenotypes of the T2 cells *in vitro*, the uninduced and induced MHC#44 cells were examined for anchorage-independent growth in semi-soft agar as described (Housey *et al.*, 1988). Rat 6 and R6#13-8 were also included for comparison. As shown in Figure 4B and Table 1, normal Rat 6 cells remained as single cells; R6#13-8 cells

formed few small colonies; while T2 transformants formed numerous large colonies. The uninduced MHC#44 cells, which constitutively expressed low levels of nmMHC-B, formed similar numbers, but much smaller colonies than the parental T2 cells (Fig. 4C and Table 1). MHC#44 cultures induced with 1 μ M and 5 μ M of muristerone A, respectively, showed significant reductions both in numbers and sizes of agar colonies. These results indicate that the induced expression of r-nmMHC-B is responsible for the partial reversion of the anchorage independent growth of the T2 cells.

Reconstitution of actin filaments in T2 cells overexpressing r-nmMHC-B.

Disruption of cytoskeletal network is one of the characteristic features of cell transformation, irrespective of the course of transformation. To examine the status of the cytoskeletal structure, we stained the actin filaments of R6, R6#13-8, T2C, and MHC#44 with FITC-phalloidin, a phalloxin that binds to filamentous actin. Like other non-transformed cells, the actin cable network was preserved in normal R6 and non-transformed R6#13-8 cells, but not in the fully transformed T2C (Fig. 5A-C). On the other hand, the MHC#44 cells, which displayed reversed transforming phenotypes, have nicely restored the actin filamentous system that was lost in the T2C cells lacking the expression of nmMHC-B (Fig. 5D). Treatment with muristerone A did not significantly enhance the actin filament bundles further in any of the test cell lines. In fact, under uninduced condition, the intensity of the actin bundles in MHC#44 seemed to be the same, except, they were less orderly compared to the actin filaments in R6 and R6#13-8 cells. This partial restoration of actin organization of MHC#44 is in line with the partial reversed transforming phenotypes described above in this report.

r-nmMHC-B suppresses tumorigenicity of T2 cells.

Data above suggested that expression of r-nmMHC-B in T2 partially reversed its transforming phenotypes, namely the cell morphology, saturation density and anchorage independent growth, and actin filaments. We therefore performed *in vivo* tumorigenicity tests in nude mice to assess the transforming status of MHC#44. Prior to the injection, MHC#44 cells were induced with or without 10 μ M muristerone A for 48 hr. To preclude any adverse effect of muristerone A on animals, T2 cells induced by same dosage of muristerone A were included in the experiment, in addition to the uninduced. At the end of treatment, cells were harvested and washed once with cold PBS, then resuspended in PBS and counted. For each group, three injection sites, each with 3×10^6 cells, were performed. Results of such experiment are shown in Figure 6 and Table 2 (Expt. I). T2 cells, treated or untreated with muristerone A, induced palpable tumors in all sites one week after the injections, and the tumors rapidly grew to sizes ranging from 2 to 7.5 cm³ by the end of the second week, when mortality began to be seen among the tumor bearing animals. In contrast, no tumor was detected at the sites injected with r-nmMHC-B expressors, either induced or uninduced. At the end of two weeks, a small palpable lump was observed at one of the injection sites with uninduced r-nmMHC-B expressors, and this was later verified by surgical examination. Suppression of malignant transformation by nmMHC-B was very striking, especially in view of the low level of nmMHC-B in the uninduced cells. To verify the anti-tumorigenic activity of r-nmMHC-B, we carried out another nude mice assay on two transfectants: MHC#44 and #33 under induced and uninduced conditions. Once again, MHC#44 produced no tumor (Table 2, Expt.

II), while T2 gave rise to large tumors within two weeks. MHC#33 induced tumors at all injection sites; however, tumor size was significantly smaller than in the control group. T2C, the backbone vectors clone, when injected into nude mice, produced large tumors ranging from 4.8 to 6.7 cm³ under both induced and uninduced situations within the same latency period as the parental T2 cells.

Discussion

In this report, we described the cloning and characterization of a rat homologue to the known nmMHC-B gene. This homologue was previously found down-regulated in transformed cells which had arisen spontaneously in the mutant p53^{val135}-overexpressing Rat 6 fibroblasts (Yam *et al.*, 1999). Upon acquiring the full length cDNA sequences, we subsequently constructed a r-nmMHC-B expression vector driven by an ecdysone-inducible promoter; the r-nmMHC-B vector was then introduced back to one of the spontaneous transformant T2 cells lacking the expression of nmMHC-B, but not of -A. The restoration of the r-nmMHC-B expression in T2 partially reversed the transforming phenotypes of T2 cells in terms of cell morphology, saturation density, and anchorage-independent growth in an ecdysone-dependent fashion. The actin network, a key feature in cell transformation, clearly reappeared, which provides a strong indication of the reversion of transforming phenotypes. Furthermore, expression of r-nmMHC-B in T2 drastically suppressed the tumorigenic activity of T2 tested in nude mice. It might seem surprising to observe such strong anti-tumor effects with both r-nmMHC-B transfectants in the uninduced condition where little leakage of r-nmMHC-B was detected, and when the difference between the T2 and uninduced MHC#44 in soft agar growth was rather mild (difference in size, but not in terms

of number of colonies); however, such discrepancy between anchorage independence growth and tumorigenicity has been documented elsewhere (for review, see Stanbridge & Wilkinson, 1980). This may well be a phenotypic trait unique to nmMHC-B-expressing cells.

In addition to the novel tumor suppression effect of r-nmMHC-B, the other intriguing finding in the current study is the unusual subcellular distribution of the ecdysone-inducible r-nmMHC-B. Endogenous nmMHC-B is evenly distributed in the cytoplasm of normal Rat 6 or untransformed R6#13-8 cells, while vectored nmMHC-B is distinctly localized in the nuclear membrane of MHC#44, becoming even more prominent in the presence of inducer (Fig. 3C). According to the study in xenopus (Kelly *et al.*, 1996), nmMHC-B is present in the cell cortex and diffusely distributed in the cytoplasm; this concurs with our observation in Rat 6 and R6#13-8 cells. During prophase, the nmMHC-B distributes to the nuclear membrane. However, unlike the finding in our study, they reported that xenopus nmMHC-B resumed its interphase localization by metaphase, while our vectored r-nmMHC-B distributed to the nuclear membrane at all stages of cell cycle. It was suggested by the authors that nmMHC-B may be involved in nuclear envelope breakdown in prophase. Along this line, it is generally believed that both nmMHC-A and -B, together with actin filaments, are likely the crucial components of the contractile ring during cytokinesis. Nevertheless, the growth rates of both nmMHC-B- expressing cells and depleted cells are the same (Fig. 4A). Later, we performed flow cytometer analysis of cell cycle on both cell lines and found no significant difference, indicating that the overexpression of nmMHC-B had no adverse effect on cell cycle progression (data not shown).

Although, prior to our current study, there has been no report of tumor suppression activity of nmMHC (or by any member of the myosin family) (Hall, 1998), numerous studies have revealed that malignant transformations are often accompanied by disruption in cytoskeletal and adhesion proteins including actin, tropomyosin, vinculin, α -actinin. Restoration of the cytoskeletal network through gene transfer could reverse the transforming phenotypes, i.e. cell shape, cell adhesion, saturation density, anchorage- independent growth and tumorigenicity (Glück *et al.*, 1993; Fernández *et al.* 1992, Boyd *et al.*, 1995; Gimona *et al.*, 1996). Because myosin is a key member in the actin microfilament network, it is conceivable, and was also shown in our study, that ectopic expression of myosin could alter the growth properties and tumorigenicity in a way similar to those observed with other actin-dependent proteins. At this point, whether reappearance of actin bundles is simply a marker for a reversed transforming phenotype, or whether it is a direct result of r-nmMHC expression is not known; however, few reports have pointed out that myosin plays a primary role in the alignment of actin filaments, and, conversely, that inactivation of the protein results in disassembly of actin organization (Lamb *et al.*, 1988; Honer, 1988; Fukui *et al.*, 1990). Along this line, recent study again showed that microinjection of myosin into REF 52 rat fibroblasts initiated the formation of actin filament bundles in the lamellae (Verhovsky *et al.*, 1995). Thus, reexpression of myosins in T2 cells might indeed trigger assembly of actin filaments, which in turn would alter the transforming phenotypes of the cells.

Despite the clear association between tumor suppression and the assembly of cytoskeletal proteins and of related adhesion proteins in general, the underlying mechanism of the suppression remains obscure. Recent studies on oncogenes and

tumor suppressor genes and their roles in signal transduction have revealed the link between adhesion plaques and key signaling pathways where various tumor suppressor genes and oncogenes are strategically located and where they coordinate rigorous checks and balances on cell signaling for growth and differentiation (for review see Hunter, 1997). Most recently, an emerging theme in the field of cytoskeletal research is that the ability of Rho/Rac family proteins in regulating the actin cytoskeleton through direct targeting of the myosin light chains, which regulate the myosin heavy chains which in turn regulate the assembly of the actin-myosin stress fiber and focal adhesion (Gimona *et al.*, 1996; Sanders *et al.*, 1999). These studies provide us a clue as to the molecular basis of the role of myosin in the actin assembly in T2 overexpressing r-nmMHC-B.

As mentioned earlier, overexpression of p53^{val135} did not readily induce transformation; rather, it induced instability of the host cells (reflected by their high spontaneous transformation rate). T2 cell line was one of the spontaneously derived transformants that exhibited altered gene expression identified by the use of mRNA differential display technique (Yam *et al.*, 1999). Being one of the two genes found suppressed in T2 cells, the r-nmMHC-B is now also found to possess tumor suppression activity. While the main focus of the current was on the effect and the possible mechanism of reexpression of the myosin gene, at the same time, it is equally important to the understanding of the initial cause of the suppression of r-nmMHC-B as well as the contribution of the p53^{val135} in the transition from non-transformed to transformed cells. At present, the promoter activity of r-nmMHC is being investigated in both R6#13-8 and T2 cells in our laboratory. Based on what is known in the field, p53 is neither a key component of spindle

assembly, nor does it play a direct role in the Rho/Rac signaling pathway through which the actin stress fibers form (Sablina *et al.*, 1999; and Esteve *et al.*, 1998).

Lastly, it is worthwhile to mention that down-regulation of nmMHC-B was initially observed in, but not necessarily restricted to, p53^{val135}-transformed Rat 6 cells. As reported recently (Yam *et al.*, 1999), suppression of the nmMHC-B was also observed in Rat 6 cells transformed by *c-H-ras*, and partial suppression was also observed in *v-myc*, but not in those transformed by *v-src*, nor in mouse SVT2 cells expressing high levels of normal p53 gene. It would be interesting to explore whether the tumor suppression effects of nmMHC-B can be applied to malignant transformations induced by oncogenes other than mutant p53. Such study should help to advance our understanding of the cellular function of nmMHC.

Materials and Methods

Cells and cell culture conditions.

The Rat 6 embryo fibroblast cell line is a subclone of an immortalized F2408 rat embryo cell line (Freeman *et al.*, 1967; Hsiao *et al.*, 1986). The R6#13-8 cell line is a derivative of the Rat 6 line infected with the retroviral vector pCH#13 carrying a temperature-sensitive mutant p53^{val135} and the bacterial neomycin gene. R6#13-8 cells yield high levels of spontaneous transformation in prolonged cultures. Clone T2 is one of the spontaneous transformants isolated from R6#13-8 cells (Yam *et al.*, 1999). The R6#8-2 cell line, a Rat 6 derivative expressing a retroviral backbone vector (Hsiao *et al.*, 1992), was included in the experiment as a control cell line. Both Rat 6 and R6#8-2 are anchorage independent and negative in nude mice assay (Hsiao *et al.*, 1986; Housey *et al.*, 1988). All cell

lines were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10% calf serum (CS), penicillin, and streptomycin at 37°C in a humidified incubator with 5% CO₂ in air.

cDNA library screening and DNA sequencing.

A 351-bp rat cDNA fragment corresponding to nucleotides 7107-7457 of human nmMHC (Phillips *et al.*, 1995) was used as a probe to screen a rat brain cDNA library (Stratagene). 5×10^5 plaques were screened. Hybridization was carried out at 42°C in 50% deionized formamide, 10% dextran sulfate, 1% SDS, and 1 M NaCl. After three rounds of amplification, positive clones were selected and sequenced at both ends using an AutoRead™ sequencing kit (Pharmacia). Complete DNA sequencing was performed on both strands.

Construction of expression vector and stable transfectants of r-nmMHC-B.

A cDNA of 6.6 kb containing the entire open reading frame and partial 5' and 3' untranslated sequences of rat nmMHC-B was subcloned into an ecdysone inducible mammalian expression vector, pIND/Hygro (Invitrogen) at NotI and ApaI sites. The resulting expression vector, pIND/nmHMC-B, was co-transfected with the pVgRXR (Invitrogen) carrying bacterial zeocin gene using LipoFectAMINE Plus reagent (BRL). The stable transfectants were selected by cloning rings in a medium containing both hygromycin B (400 µg/ml) and zeocin (300 µg/ml).

Antibodies and western blot analysis.

Confluent cultures in 60 mm dishes were washed with cold PBS three times and lysed in 400 μ l NET buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 5 mM EDTA, pH 8.0, 1 mM APMSF, 1 μ M E-64, 1 μ M pepstatin, 100 mM NaVO₅, and 10 μ g/ml aprotinin) plus 1% NP-40 on ice according to Lu *et al.* (1992). For western blotting analysis, 10 μ g of protein extracts were loaded on a 5% SDS-PAGE. After separation, the proteins were transferred to an Hybond-C nylon membrane (Amersham), reacted to rabbit anti-human nmMHC-A and -B polyclonal antibodies (supplied by Dr. R.S. Adelstein) and visualized by the ECL detection kit (Amersham) according to the instruction manual.

Immunofluorescent staining.

Immunofluorescent staining was performed according to Martinez *et al.* (1991) with slight modification. Cells grown on 1% gelatin-coated coverslips were rinsed three times in ice-cold PBS. Cells were fixed in cold methanol for 5 min. Antibodies specific to human nmMHC-A and -B chains (supplied by Dr. R.S. Adelstein) were employed for immunofluorescent staining. Specimens were mounted on glass slides with a mounting solution (20% glycerol in PBS).

Growth curves and muristerone A treatment.

Cells were seeded at 1.7×10^4 cells per 35 mm plate in triplicate and grown in DMEM plus 10% CS medium. The following day, seeded cultures were given different concentrations of muristerone A. Cell numbers were counted every other day. Mean values and standard deviations of the triplicates were calculated and plotted against time.

Soft agar assay.

Tested cells were treated with or without muristerone A for 48 hr, then suspended at 1×10^4 /60 mm plate with 2 ml of 0.4% Bacto-agar in DMEM containing 20% fetal calf serum (FCS) in the absence or presence of 1 or 5 μ M of muristerone A and overlaid above a layer of 5 ml of 1% agar in the same medium in triplicate plates. Cells were refed with a top agar medium biweekly. At day 28, colonies were stained with the vital stain 2-(p-iodopenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride hydrate (INT) for 48 hr at 37°C in a 5% CO₂ incubator. The number of colonies was scored using the Eagle Eye ®II System (Stratagene).

Labelling of actin filaments.

The cells were grown on coverslips were fixed with 3.7% formaldehyde in PBS for 10 mins at room temperature, then permeablized with 0.2% Triton X-100 in PBS for 1 min at room temperature, washed three times with PBS. The cells were stained with FITC-phalloidin (Sigma) at 5 μ g/ml in PBS for 30 mins at 37°C, then washed with PBS before mounting on microscopic slides with a drop of anti-photobleaching Mowiel 4-88 (Calbiochem, USA) for examination under fluorescent microscope.

Nude mice assay.

For the *in vivo* tumorigenicity test, confluent cultures of tested cells were either induced or uninduced with 10 μ M muristerone A for 48 hr. At the end of treatment, cells were harvested and washed with cold PBS once, then resuspended in fresh PBS and counted for cell numbers. Triplicate s.c. inoculations with

3×10^6 cells were performed. Experiments were monitored over a two-week duration.

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Figure Legends

Figure 1. Expression of A- and B-isoforms of nmMHC in T2 and R6#13-8 cells. **(A)** Immunoblotting analysis: cell lysates were extracted from Rat 6, the parental cell line; R6#13-8, the untransformed Rat 6 clone overexpressing p53^{val135}; and T2, the spontaneous transformant derived from R6#13-8 cells. Ten μg of each lysate were loaded per lane and separated by SDS-PAGE. The immunoblots were hybridized with anti-nmMHC-A and -B antibodies. **(B)** Immunofluorescent staining: cells were grown on gelatin-coated coverslips, fixed and incubated with anti-nmMHC-A and -B antibodies. Texas Red- and FITC-conjugated secondary antibodies were used in conjunction with nmMHC-A or -B antibodies respectively. Cells were observed under a fluorescent microscope at 100 \times magnification. Cos7 cells only express B-isoform and human platelets only express A-isoform.

Figure 2. Alignment of amino acid sequences of r-nmMHC-B (Rat) (GenBank AF139055) with those of human (Hum) (GenBank M69181) and chicken (Chk) (GenBank M93676) nmMHC-B. Identical amino acid residues are indicated by dashes and amino acid residues different from that of r-nmMHC-B are shown. Locations of the two insertions in chicken nmMHC-B are indicated by the arrowheads ($_$). The amino acid sequences of the two insertions are PESPVPVKHQ (insert after residue 211) and EIQNIQRACFYDNITGLHDPP (insert after residue 621). The ATP binding site (a.a. 159-194), actin binding site, 28-residue repeat (a.a.857-884) of the α -helical coiled core are underlined. The α -helical core domain (a.a. 843-1933) is marked by the arrowbars.

Figure 3. Characterizations of MHC-B#33 & MHC-B#44 clones expressing the acquired nmMHC-B gene. **(A)** Western blot analysis. Cells were grown to subconfluency in 60 mm plates. Each group was treated with 0, 1, 2, 5 or 10 μM of muristerone A for 48 hours before harvesting for cell lysate. Ten μg of cell lysate for each sample were loaded per lane and separated on SDS-PAGE and the resulting blot was reacted to anti-nmMHC-B antibody, detected by ECL detection system (Amersham). Rat 6, R6#13-8, T2, T2C cells are included for comparisons. **(B)** Cell morphology. All tested cell lines were maintained in growth medium in the absence of muristerone A. Photographs were taken under the phase contrast field of a Zeiss Axiophot microscope at 40 \times magnification: **(C)** Immunofluorescent staining. Semi-confluent cultures grown on coverslips were treated with or without muristerone A for 48 hrs, then fixed with cold methanol and immunostained with anti-MHC-B antibody followed by FITC-conjugated anti-rabbit antibody. a, b, c & e : no treatment; d & f: treated with 5 μM muristerone A for 48 hr. Photographs were taken under a fluorescent Zeiss Axiophot microscope using 100x objective in oil immersion.

Figure 4. Comparison of growth properties of T2 and MHC#44 cells. **(A) left panel:** Growth curves of MHC#44 cells treated with various dosage of muristerone A. Cells at 1.7×10^4 were seeded in six-well plates. Next day, cells were maintained in a growth medium supplemented with designated concentrations of muristerone A throughout the entire experiment. Triplicate

wells were used for each time point. Mean values of triplicates are calculated and plotted in semi-log graph. Error bars indicate standard deviations. A “8 day” time point of T2 cells in the absence or presence of inducer is also included. **(A) right panel:** Dosage response curves of T2 & MHC#44 upon treatment of muristerone A. The growth of each treatment group was presented as percent of growth relative to its respective no-treatment control. **(B)** Anchorage-independent growth. 1×10^4 cells were seeded in 0.4% top agar containing DMEM plus 20% fetal calf serum. Cells were refed with top agar medium supplemented with each designated concentration of muristerone A at day 14. Experiments were terminated on day 28 and stained with INT-vital stain. Colonies were counted and photographed with an Eagle Eye ®II image system (Stratagene). Soft agar colonies obtained from each group were calculated and presented as table form. Mean values and standard deviations of triplicates of each group are shown. **(C)** Representations of soft agar colonies of T2 and MHC#44 cells with and without the inducer. An average size of the colonies derived from each group is displayed in this panel. The scale bar in white color is 0.1 mm in length.

Figure 5. Localization of actin filament bundles in various cells using FITC-conjugated phalloidin. A. Rat 6 cells, B. R6#13-8, C. T2C, and D. MHC#44 cells. Photographs were taken under a fluorescent Zeiss Axiophot microscope using 100x objective in oil immersion.

Figure 6. Comparison of the tumorigenicity of T2 and MHC#44 in nude mice. Cells were treated with 10 μ M muristerone A for 48 hrs and washed with PBS. Each group of cells was inoculated at 3×10^6 per site. Experiments were carried out for two weeks. Pictures were taken two week after the animal received injections.

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