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*Full Length Research Paper*

# Mutated N-ras does not induce p19$^{arf}$ in CO25 cell line

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**The mouse cell line (CO25) used in this study was transfected with a glucocorticoid inducible mutated human N-*ras* oncogene under transcriptional control of the steroid-sensitive promoter of the mouse mammary tumors virus long terminal repeat MMTV-LTR. This study was aimed to investigate the expression of p19$^{arf}$ and MDM2 genes under the effect of N-ras oncogene induction and to invent the role of p19$^{arf}$, MDM2 in N-ras pathway during various periods (12, 24, 48, 72, 96 h) using western blotting method. The levels of β-actin proteins in the same periods were our control group. The observations showed no increase of p19$^{arf}$ protein expression in normal, cancer and differentiated CO25 cells. MDM2 was accumulated until 72 h and after 96 h, it showed a dramatical decrease while β-actin levels were increased correlated to the volume of protein loaded to the gel. Because of the role of p19$^{arf}$ as tumor suppressor and p53-MDM2 linker, it is highly recommended to investigate the relationship between N-ras and p53 and MDM2 in the same system to recognize the molecule that may play a linker molecule between p53 and MDM2 in p19$^{arf}$ lack system.**

**Key words:** Oncogene, N-ras, p19, myoblast, CO25 cells, differentiation, MDM2.

## INTRODUCTION

The ras oncogene has been shown to affect differentiation in various cell types in different ways, by inducing the resistance retinoic acid, which is a potent effectors of epithelial cell growth, differentiation (Olson et al., 1987) and neurogenic differentiation (Muroya et al., 1992). Expression of transforming *ras* genes in different myoblast cell types inhibited myogenic differentiation by blocking or down regulating the expression of transcription factors *MyoD*1 and myogenin (Lassar et al., 1989). N-*ras* and H-*ras* expression in C2 and CO25 cells blocked the induction of muscle specific proteins α-actin, desmin, myosin heavy chain, and also led to expression of non muscle protein β-and γ-actin and vimentin (Olson et al., 1987; Gossett et al., 1988). In mouse myogenic cells 23A2, the activated H-*ras* gene prevented muscle differentiation, and caused two to three fold more protein kinase C (PKC) activity than wild type myofibers (Vaidya et al., 1991, Payne et al., 1987). *Ras* oncogene family act as a molecular switch in signal transduction from growth factor receptors on the cell surface to effectors molecules because it is located on the inner side of the plasma membrane, and activated by growth factors (Downward, 1992; Li et al., 1995). It is suggested that *ras* activated kinases (MAP kinases) phosphorylate transcription factors and thereby modify their ability to induce gene expression (Pawson and Hunter, 1994).

In this article, p19$^{arf}$ but not p16$^{INKa4}$ was used because CO25 cell line is mice original cells. p19$^{arf}$ is tumor suppressor gene located in INK4a gene locus and known as alternative reading frame (ARF); it consists of 169 amino acid, and is considered as a product of alternative splicing of mRNA involved in blocking of cell proliferation (Pamero et al., 1998). p19$^{ARF}$ up-regulates function of the *p53* tumor suppressor protein by inhibiting Mdm2 activity

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(Pomerantz et al., 1998; Zhang et al., 1998; Stott et al., 1998). So, *p19*$^{ARF}$ plays a key role in some cell activities like tumor suppression (Stanchina et al., 1998) and blocking of cell proliferation by linking p53 to ras (Pomerantz et al., 1998). In the light of these information, ARF appears to play a more relevant role as a pro-apoptotic factor via inhibition of MDM2, a ubiquitin ligase that destabilizes the tumor suppressor p53 protein (Kim and Sharpless, 2006; Gonzalez and Serrano, 2006).

In this investigation, we tried to identify if p19$^{arf}$ oncogene has any role in the mutated N-ras pathway or not. So to achieve this goal, we used CO25 myoblast cell line.

The mouse cell line (CO25) used in this study had been transfected with a glucocorticoid inducible mutated human N-ras oncogene under transcriptional control of the steroid-sensitive promoter of the mouse mammary tumors virus long terminal repeat MMTV-LTR.

When the cells grow in a weakly mitogenic medium, they proceed to form myotubes after four days, and fail to differentiate when they grow in the presence of dexamethasone (Dex), displaying characteristics of a transformed phenotype. So, CO25 cells was chosen in this work as an attractive model system since it allows the identification of differentiated, transformed and normal cells which can be clearly distinguished from their differentiated counterparts which form multinucleated myotypes as shown in different figures given in this work. C2 cell line was established from primary cultures prepared from injured mouse thigh muscle (Yaffe and Saxell, 1977). The CO25 cells were derived by transfection of the C2 cells with a plasmid containing mutationally activated human N-ras gene in codon 61under transcriptional control of steroid-sensitive promotor of the mouse mammary tumor virus long terminal repeat (MMTV-LTR) (Gossett et al., 1988). Dexamethasone (Dex) treatment of the cells leads to the induction of the *N-ras* gene and the development of transformed phonotype. Differentiation is inhibited in (10% HS) horse serum, which is mitogen deficient medium. The cells transferred to 10% foetal calf serum (FCS) started to fuse and form multinucleated myotubes but both C2 and CO25 proliferated in 10% (FCS). Changes in the morphology of the cells either during differentiation or the transformation process enabled us to monitor the stages in either process.

## MATERIALS AND METHODS

### Cell culture

The C025 cell line (gift from Dr. E.N. Olson, University of Texas) was maintained in DMEM (Gibco Ltd. Paisley Scotland) supplemented with 20% FCS (Gibco), 1% L-glutamine and penicillin/ streptomycin (100 units/ml). Cells were gassed with 10% $CO_2$/ 90% air and incubated at 37°C in $CO_2$ Incubator. To initiate differentiation, cultures at about 80% confluence were transferred to the same medium containing 10% HS (Gibco) as a fusion promoting medium, instead of FCS. After four days (96 h) of incubation, formation of myotubes was observed (Figure 2). To induce expression of the N-ras oncogene, the cells maintained in the 10% HS were exposed to 1µM dexamethasone for various periods of time (12, 24, 48, 72, 96 h).

### Light microscopy

Phase-contrast observation was carried out using a Nikson TMS inverted microscope fitted with a Nikon F-301 camera for photography. Kodak Tri-X-pan 400 film was used.

### Growth curves

CO25 cells were seeded at a concentration of 1 to 2 × $10^6$ cells in a 25 $cm^2$ flask. This was carried out in triplicate. To harvest, the medium was removed, 2 ml trypsin-EDTA (ethylene diamine tetracitic acid) (0.2:0.04) solution was added and incubated at 37°C for approximately 5 min or until the cells were observed to be lifted from the flask. Addition of 2 ml medium inhibited any further breakdown of the cells. 0.5 ml of cells was added to 19.5 ml 0.9% saline solution containing 0.5% formaldehyde and counted with the aid of a Coulter counter (Coulter Electronics Limited.).

### Cell extraction

When the cells reached 80% confluence, the medium was changed to fusion promoting medium with or without 1µM dexamethasone for 12, 24, 48, 72, and 96 h periods of time. The monolayer cells was rinsed twice with ice-cold PBS and then lysed in 0.5 ml of extraction buffer containing (1% NP-40, 50 mM Tris Base, pH 8.0, 50 mM NaCl, 50 µg/ml Leupeptin and 1 mM Phenylmethyl sulphonyl floride (PMSF). The cells were transferred to a 1.5 ml Eppendrof tube after incubation on ice for 20 min with occasional rocking, vigorously vortexed for 10 s and then centrifuged at 1300 *g* for 10 min at +4°C. Supernatant was used for the analysis. Total protein was estimated using the method by Bradford (1976).

### Electrophoresis and blotting

For each sample, 30 µg of protein in 20 µl volume was mixed with 5 µl 0f 4X SDS sample buffer (0.4 M Tris, 0.4 M dithiothreitol, 8% SDS, 40% glycerol and 0.04% bromophenol blue, pH 6.8), denatured at 100°C for 2 to 3 min and then resolved on a 12% SDS-ployacrylamide mini gel. The rainbow molecular weight marker (Amersham) was prepared in the similar way to samples and loaded parallel well on the gel. Electrophoresis was performed at 80 V and 150 V for the stacking and the resolving gel respectively, using a Tris-Glycine electrophoresis buffer (0.25 M Tris, 1.92 M Glycine, pH 8.5). Proteins were transferred to 0.45 µM nitrocellulose (NEN Research) in LKB Multiphor II apparatus using the Tris-Glycine buffer containing 20% methanol at a current of 15 V for 30 min and then the blot was air-dried.

### Detection of proteins

After well washing in a dark room, the blot was drained, and then incubated in a 1:1 mixture of enhanced chemiluminescence (ECL) Western Blotting detection reagents A and B (Amersham) for 1 to 2 min. The blot was exposed to Kodak X-ray film for 3 to 60 s.

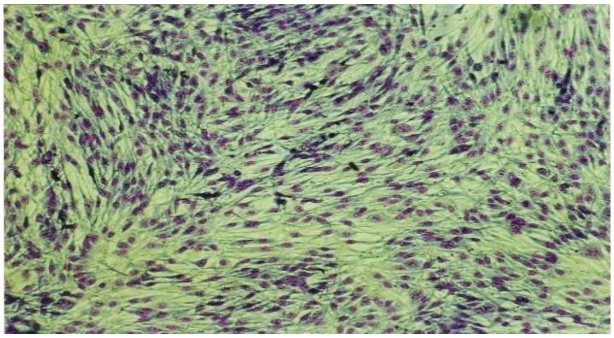


**Figure 1.** General view of CO25 cells in the growth medium of 20% FCS after 4 days. Magnification 100×. Gimsa and May-grunwald stain were used (Sigma).

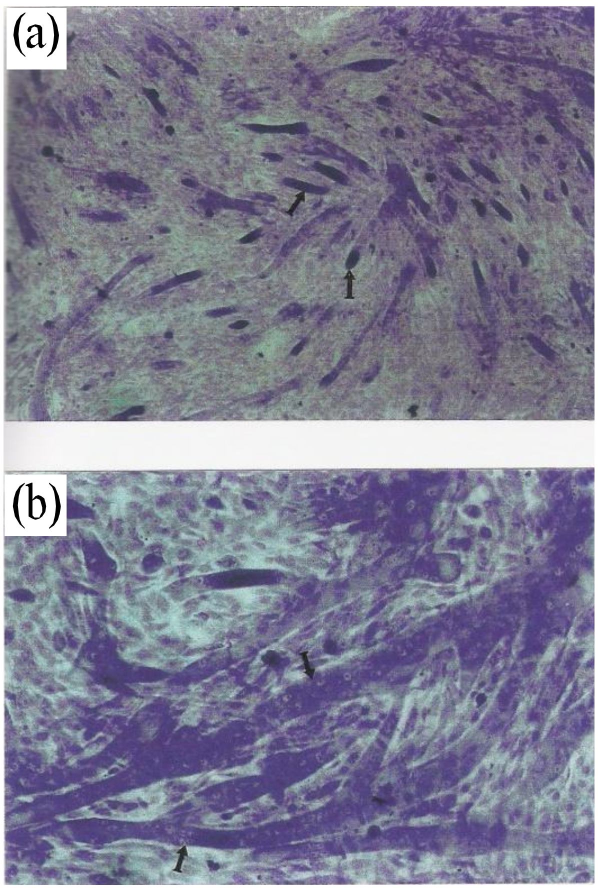



**Figure 2.** Morphology of CO25 myoblast cells grown in 10% HS (a) after 4 days to form myotubes, and after 8 days, myotubes were clearly formed (b). Magnification 100×. Gimsa and May-Grunwald stain were used (Sigma).

#### Immunolabelling

The blot was blocked for 1 h in the rinse buffer (150 mM NaCl, 20 mM Tris-HCl, pH 7.4, 0.02 Tween-20). The blot was blocked for 1 h in the rinse buffer containing 5% skimmed milk and probed for 2 h in the same buffer containing 1:100 monoclonal antibody to $p19^{ARF}$, β-actin (Amersham). The blot was washed four times for 5 min in the rinse buffer, reblocked for 10 min in the blocking buffer and incubated with a Rabbit anti-mouse Ig (HRP) (Dako Limited) and finally, twice for 10 min in the rinse buffer without Tween-20.

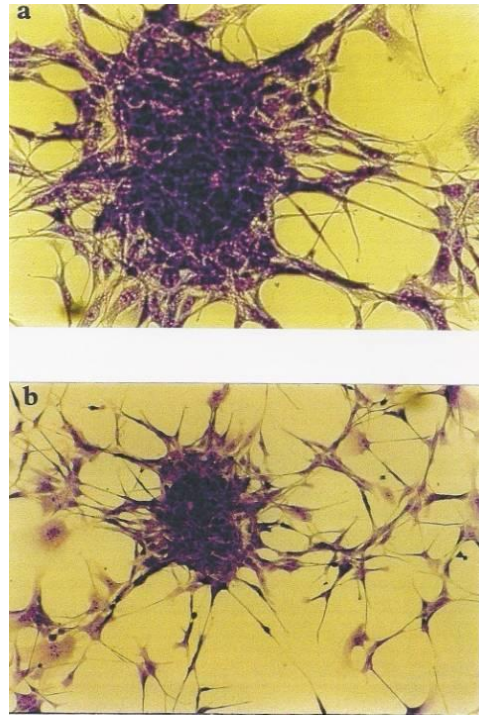



**Figure 3.** Morphology of CO25 myoblast cells grown in 10% HS+Dex. (a) Foci formation after 6 days. (b) Foci formation after 4 days. Magnification 100×. Gimsa and May-grunwald stain used (Sigma).

## RESULTS AND DISCUSSION

The ability of CO25 myoblast cells transfected with a plasmid containing steroid –inducible mutated N-*ras* oncogene MMTV-LTR was studied in different situations where cell differentiation was promoted by the activation of the N-*ras* oncogene. The cells grown in 20% FCS showed a high proliferation rate as confirmed by the results collected from cells morphology (Figure 1) and growth curve (Figure 7) whereas, CO25 cells differentiated into myotubes in the presence of 10% HS (Figure 2a). The results demonstrate clearly that the expression of an activated mutant N-*ras* oncogene suppressed the ability of CO25 myoblast to form myotubes as seen in Figure 2b. The inhibition of myogenic differentiation by the N-*ras* oncogene was reversible by removing dexamethasone. Expression of N-*ras* oncogene under the effect of dexamethasone induced CO25 cells to make foci (Figure 3a and b). All of these results are compatible

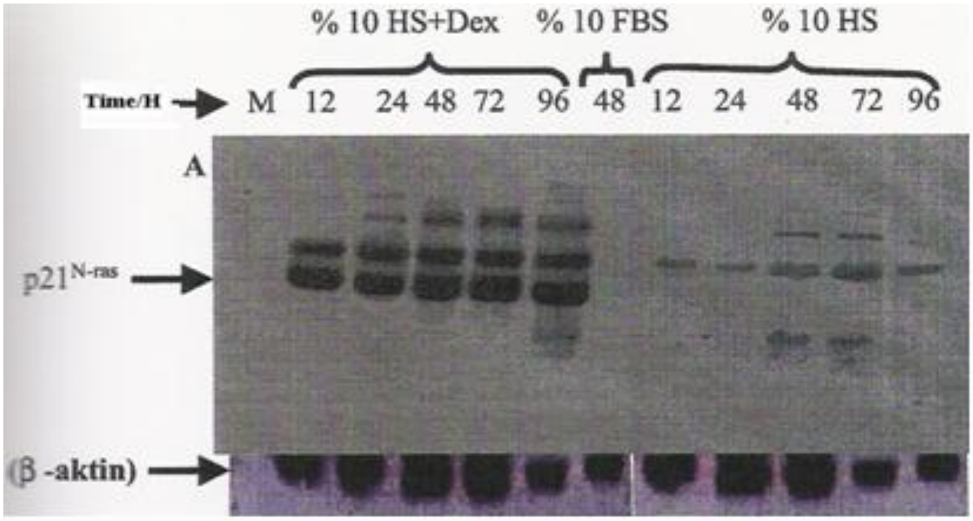



**Figure 4.** Expression of N-ras protein in CO25 myoblasts bearing different mediums and different time periods determined by Western Blotting analysis at 5 s exposure time.

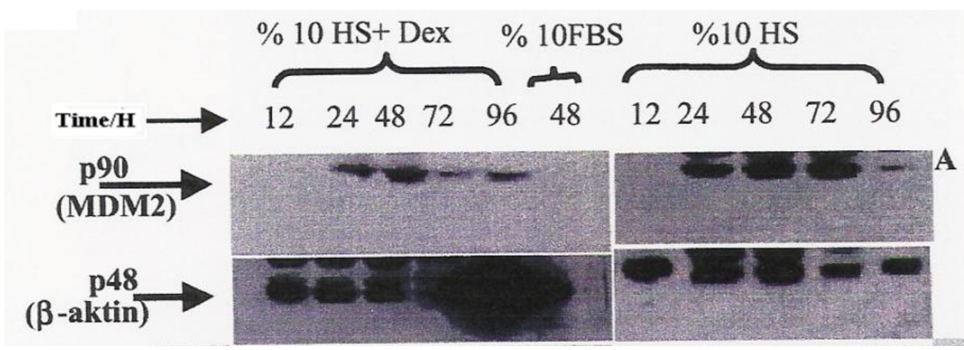



**Figure 5.** Expression of MDM2 protein in CO25 myoblasts bearing different mediums and different time periods determined by Western Blotting at 5 s exposure time.

with the findings of Gossett et al. (1988). At the same time, these observations confirmed that our system worked well and in the right direction. Many researchers suggested that *ras* oncogenes inhibited the myogenic differentiation in various myoblast cell types by preventing accumulation of regulatory factors like, mck, MyoD1, and Ach, which are required for the transcriptional induction of muscle-specific genes muscle cell line (Olson et al., 1987; Gossett et al., 1988; Honda and Yasuda, 1999).

In this study, N-ras protein levels were investigated in normal, differentiated and transformed cells by Western Blotting techniques (Figure 4).

Pamero et al. (1998) reported that *p19$^{ARF}$* links the tumor suppressor *p53* to *ras*. At the same direction, Honda and Yasuda (1999) confirmed that the association of p19$^{ARF}$ with MDM2 inhibits ubiquitin ligase activity of MDM2 for tumor suppressor p53. But when these researchers investigated the relationship between MDM2-p19$^{ARF}$-p53 in lymphomagenesis, they found that the level of p19$^{ARF}$ was not dependent on the level of MDM2 all the time. These results are compatible with our observations. In normal 20% FCS medium, MDM2 was absent, but in 10% HS, MDM2 was accumulated until 72 h and after 96 h, it showed a dramatical decrease (Figure 5). At the same time, p19$^{ARF}$ did not appear in each of them (Figure 6). While the deficiency of p19$^{ARF}$ reduces macrophage and vascular smooth muscle cell apoptosis, aggravation of atherosclerosis was suggested by Gonza´lez-Navarro et al. (2010) which may mean that p19$^{ARF}$ has a negative role in myoblast cells differentiation. This study confirmed these observations in CO25 cells. These data indicate the possibility that p19$^{ARF}$ may function independent on MDM2 and may involve other unknown interacting

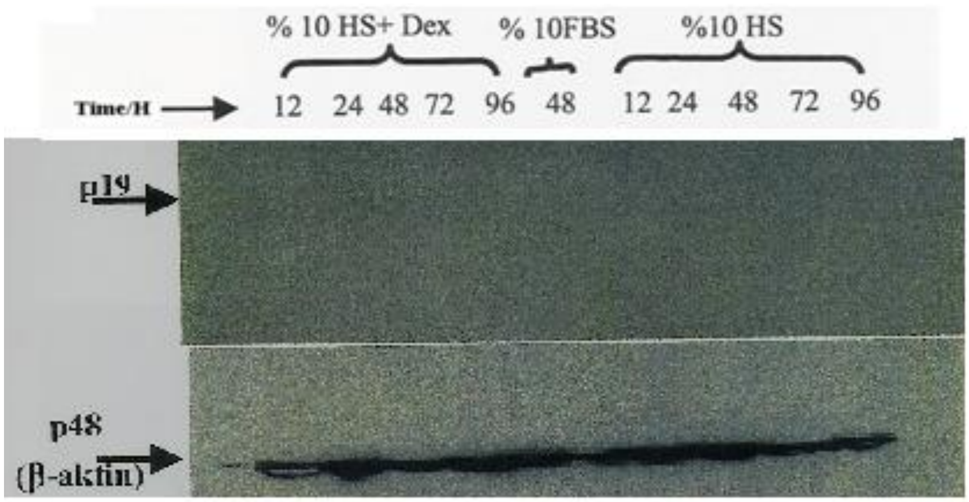
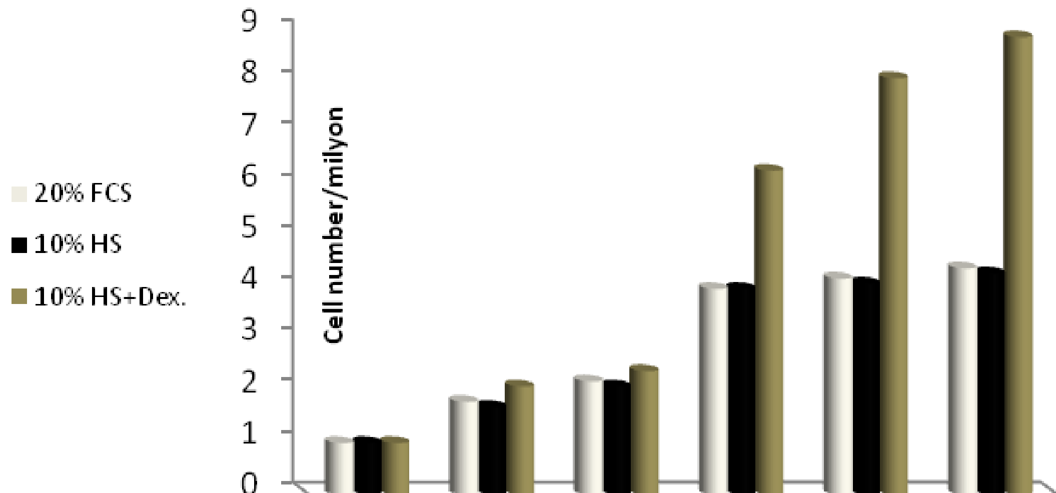



**Figure 6.** Expression of p19$^{ARF}$ protein in CO25 myoblasts bearing different mediums and different time periods determined by Western Blotting analysis as described in materials and methods, at exposure time of 15 s.




| Time (h) | 0 | 12 | 24 | 48 | 72 | 96 |
|---|---|---|---|---|---|---|
| 20% FCS | 1 | 1.8 | 2.2 | 4 | 4.2 | 4.4 |
| 10% HS | 1 | 1.7 | 2.1 | 4 | 4.1 | 4.3 |
| 10% HS+Dex. | 1 | 2.1 | 2.4 | 6.3 | 8.1 | 8.9 |

**Figure 7.** Growth rate of CO25 cells in growth medium (20% FCS), in fusion promoting medium (10% HS), and in the presence of 1mM Dexamethasone in 10% HS. Values represented the average of duplicate flasks from two separate experiment.

partners. Contrary to the observations of Pamero et al. (1998) and Honda and Yasuda (1999), our observations showed that p19$^{arf}$ has no linking role in N-ras pathway at CO25 cells because p19arf was absent in all levels but MDM2 levels were increased during 12, 24 and 48 h. To confirm these results, it is highly recommended to investigate p53 levels under N-ras activation pathway.

## ACKNOWLEDGEMENT

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