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Association of castration-dependent early induction of *c-myc* expression with a cell proliferation of the ventral prostate gland in rat

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Abbreviations: EDTA, ethylene diamine tetraacetic acid; SDS, sodium dodecyl sulfate; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling

Abstract

The protooncogene *c-myc* is known to be associated with both cell proliferation and apoptosis. The possible cellular affects of castration on the ventral prostate gland of rat as well as the relationship to a castration induced *c-myc* expression were examined. Levels of *c-myc* mRNA in the ventral prostate gland peaked at 6 h (early induction) and 48 h (late induction) after castration, respectively. Castration-induced DNA fragmentation was not observed at an early induction of *c-myc* mRNA. DNA fragmentation appeared to be testosterone-dependent. On the other hand, cellular DNA synthesis measured by [^{3}H]thymidine uptake in the ventral prostate gland was increased to maximum at 6 h after castration. These results suggest that an early induction of *c-myc* mRNA in ventral prostate gland after castration is closely associated with cell proliferation of the gland.

Keywords: apoptosis, castration, *c-myc*, prostate

Introduction

The prostate gland of an adult male is dependent on androgen for its growth and function. Androgens have effects on prostatic cell proliferation or death (Isaacs, 1984). Testosterone is irreversibly converted *via* 5α-reductase to dihydrotestosterone within the prostate (Bruchovsky *et al*., 1968). After castration, the level of prostatic dihydrotestosterone decreases below a critical threshold and the prostate rapidly regresses (Kyprianou *et al*., 1988; English *et al*., 1989). Recent studies showed that the involution of the normal prostate gland induced by androgen ablation is an active process involving a cascade of specific biochemical steps that lead to the programmed death of the androgen-dependent glandular epithelial cells (Kerr *et al*., 1973; Sandford *et al*., 1984; Kyprianou *et al*., 1988). Castration-induced death of prostatic epithelial cells is accompanied by specific gene expression, *i.e. c-myc* (Quamby *et al*., 1987; Buttyan *et al*., 1988), p53 (Colombel *et al*., 1992), and IGFBP (Nickerson *et al*., 1998). c-Myc protooncogene appears to play an important role in the regulation of cell growth. A transient increase of *c-myc* mRNA occurs during liver regeneration after a partial hepatectomy (Makino *et al*., 1984) and also has been demonstrated after mitogenic stimulation of quiescent lymphocytes (Kelly *et al*., 1983). Conversely, *c-myc* mRNA was increased in the involution of the prostate after castration in rat, and the induction of *c-myc* mRNA was correlated with apoptosis of the ventral prostate (Quamby *et al*., 1987). Recently, Lee *et al*. reported that *c-myc* mRNA is transiently induced at the beginning of a period after castration (Lee *et al*., 1999). In this study, we present a strong evidence that an early induction of *c-myc* mRNA in the ventral prostate after castration may be correlated with proliferation of the ventral prostate.

Materials and Methods

Materials

Testosterone, agarose, piperazine-N,N'-bis[2-ethanesulfonic acid] (PIPES), 3-[N-morpholino]propanesulfonic acid (MOPS), formaldehyde, formamide, ethidium bromide, phenol, and sodium dodecyl sulfate (SDS) were purchased from Sigma Chemical Co. (St. Louis, MO). Random primed DNA labeling kit, terminal transferase and guanidine thiocyanate were purchased from Boehr-

inger Mannheim (Indianapolis, IN). α-[^{32}P]dATP (specific activity 3,000 Ci/mmol), α-[^{32}P]dideoxy-ATP (specific activity 3,000 Ci/mmol) and [^{3}H]thymidine (specific activity 20 Ci/mmol) were purchased from Amersham (Arlington Heights, IL).

Animals and Tissue Preparations

Male Sprague Dawley rats were obtained from Korea Research Institute of Chemical Technology. Groups of mature rats (350 g body weight) were castrated and in some cases, treated with testosterone propionate (5 mg/0.1 ml in corn oil, *s.c.*). Tissues were removed immediately after decapitation.

Northern blot hybridization

Total RNA was prepared by a modification of the method of Karlinsey *et al.* (Karlinsey *et al.*, 1989). Northern blot hybridization was performed by a modification (Lim *et al.*, 1994) of Virca *et al.* (Virca *et al.*, 1990) as described. The total RNAs (50 mg) were denatured in formaldehyde and formamide, and fractionated by electrophoresis through a formaldehyde-1.2% agarose gel. RNA was transferred from the gel to a Nytran® membrane in the presence of 10 x SSC (5 M NaCl, 0.15 M Na-citrate). After baking at 80°C for 1 h, the blotted membrane was prehybridized for 30 min at 60°C in hybridization solution containing 50 mM PIPES, 100 mM NaCl, 50 mM sodium phosphate (pH 6.8), 1 mM EDTA, and 5% SDS. The membrane was hybridized with probe of 1×10^6 cpm/ml prepared by a random primed DNA labeling kit. After hybridization for overnight, the blot was rinsed twice with 1 x SSC solution containing 5% SDS for 15 min at 60°C and followed by autoradiography at -70°C using Kodak X-OMAT AT X-ray film. A *Xba* I-*Bam* HI fragment of pSV-*c-myc* was used as probe for *c-myc* (Land *et al.*, 1983). Two separate experiments were performed repeatedly.

Determination of DNA synthesis

[^{3}H]Thymidine (specific activity; 20 Ci/mmol, New England Nuclear) was used to evaluate DNA synthesis. After castration, the rats were injected intraperitoneally with [^{3}H]thymidine (20 μCi/100 g of body weight in 0.4 ml of sterile saline) and DNA synthesis was assayed by a modification (Lim *et al.*, 1993) of the method of the Barbiroli et al. (Barbiroli *et al.*, 1971).

DNA extraction and apoptotic DNA fragmentation

Total DNA was isolated from frozen tissue (Tilly *et al.*, 1993) and quantitated by absorbance at 260 nm. One microgram of DNA from each sample was labeled at 3'-ends using [α-^{32}P]dideoxy-ATP (3,000 Ci/mmol; Amersham Arlington Heights, IL) by terminal transferase enzyme (Boehringer-Mannheim, Indianapolis, IN) with modification (Cha *et al.*, 2000) as described (Tilly *et al.*, 1993). The labeled DNA samples (200 ng/lane) were loaded onto 2% agarose gel and separated by electrophoresis at 50 V. The gels were dried in a slab-gel dryer without heat and exposed to X-ray films for qualitative analysis. After autoradiography, the amount of radiolabeled dideoxy-ATP incorporated into low molecular weight (<20 kb) DNA fractions can also be quantitated by cutting the respective fraction of DNA from the dried gel and counting in a liquid scintillation counter. The amount of [α-^{32}P]-dideoxy-ATP incorporated into the low molecular weight DNA fractions per nanogram of DNA labeled was used as a quantitative estimation of the degree of apoptotic DNA fragmentation within various samples.

TUNEL assay

Apoptotic bodies were detected in formalin-fixed, paraffin embedded tissue sections using the ApoTag®Peroxidase *in situ* apoptosis detection kit (Intergen Co., NY) which is based on the terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) method. Five micromolar sections were prepared and treated according to the manufacturers instructions. Apoptotic bodies were visualized and photographed under an inverted microscope.

Results

Differential expression of *c-myc* mRNA after castration in the ventral prostate

We have previously reported that protooncogene *c-myc* is differentially regulated in the ventral prostate after castration (Lee *et al.*, 1999). To confirm a castration-dependent induction of *c-myc* mRNA, total RNAs were prepared from the ventral prostate. The presence of *c-myc* mRNA was detected by Northern blot hybridization using the third exon of the human *c-myc* gene as a probe. As a control, sham operation was used. In the ventral prostate, levels of *c-myc* mRNA increased to an approximately 10 fold at 6 h, 3 fold at 24 h and 9 fold at 48 h after castration, respectively (Figure 1). The membranes used for detection of *c-myc* mRNA were also washed and rehybridized to cDNA probes for the major androgen-dependent secretory protein (C1 subunit for prostatic binding protein) and androgenrepressed protein (TRPM-2) of the rat ventral prostate. C1 mRNA was gradually repressed and TRPM-2 mRNA was induced at 48 h after castration. These results suggest that *c-myc* gene is differentially regulated in the ventral prostate after castration.

Early induction of *c-myc* mRNA after castration is not related to apoptotic DNA fragmentation

Late induction of *c-myc* mRNA may play a role in the

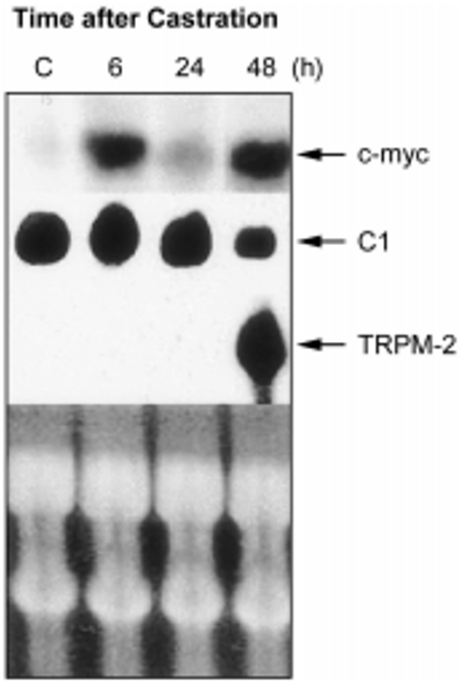

Figure 1. Time course of the castration-dependent change of *c-myc* in the ventral prostate. At the indicated time, the ventral prostate was removed from groups of rats which were either sham-operated (control) or castrated, and total RNA was prepared. Total RNA (50 μg) was analyzed by Northern blot hybridization using a *c-myc* cDNA probe as described in 'Materials and Methods'.

epithelial cell regression and apoptosis was seen in the ventral prostate after androgen withdrawal (Quamby *et al.*, 1987). To investigate whether an early induction of *c-myc* mRNA is related to apoptosis, DNA fragmentation was assayed. Analysis of DNA fragmentation of the ventral prostate using 3'-end labeling with terminal transferase indicated that the ventral prostate of intact rat contained predominantly high molecular weight DNA. Castration increased DNA cleavage into low molecular weight fragment of 185 bp multiples in a time-dependent manner with a detectable increase at 1 day and a maximal increase at 5 days after castration. As in the intact rats, the ventral prostate from an early phase (6 h) after castration did not exhibit apoptotic DNA fragmentation (Figure 2), thus ruling out the possibility that the operation procedure itself causes apoptotic cell death. TUNEL assays were also performed on the ventral prostate sections in order to confirm the correlation between DNA fragmentation and apoptotic body at the various time points after castration. In sham control and 6 h following castration, apoptotic body was not detected in the ventral prostate. By 24 h, a small number of cells were apoptotic and a number of apoptotic cells were rapidly increased by 48 h following castration in the ventral prostate (Figure 3). Therefore, the early induction of *c-myc* mRNA is not related to apoptotic DNA fragmentation. It is known that cast-

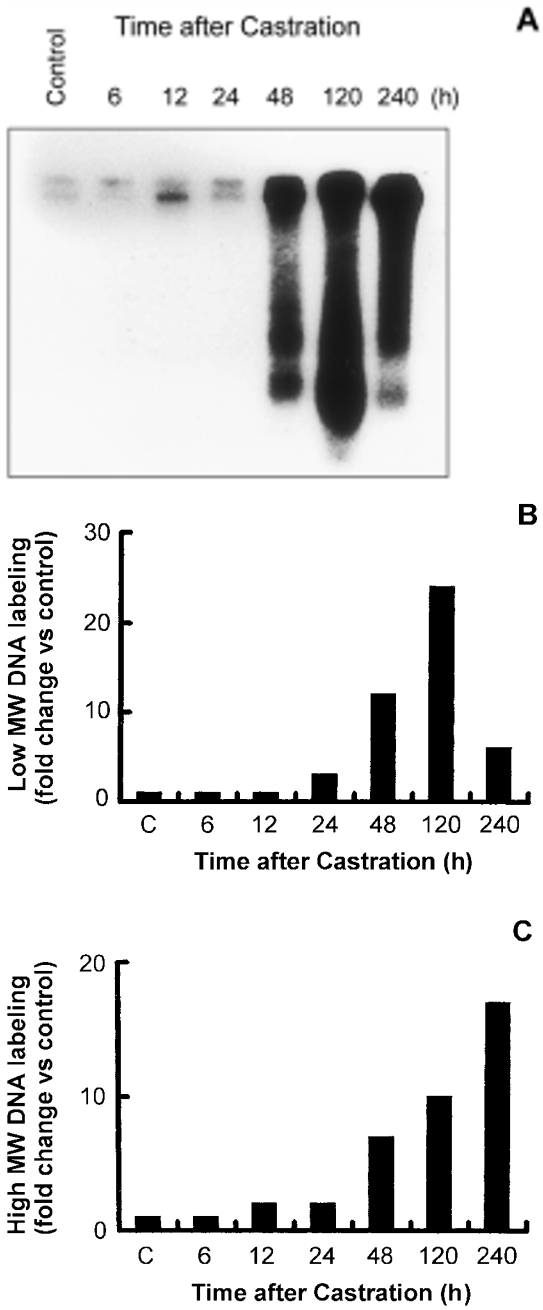

Figure 2. Castration-induced DNA fragmentation in the ventral prostate. At the indicated time, the ventral prostate was labeled on 3'-ends with-^{32}Pdideoxy-ATP, and analyzed by electrophoresis in 2% agarose gel. Autoradiography (A) and followed by β-counting of low molecular weight ($\leq$ 15 kb, B) and high molecular weight ($\geq$ 15 kb, C) DNA fractions. The amounts of α-[^{32}P]dideoxy-ATP incorporated into DNA fractions from different groups were compared with that of intact animals at each time point.

ration-dependent apoptosis of the ventral prostate is related to androgen withdrawal (English *et al.*, 1989; Kyprianou and Isaacs, 1998). To identify whether testosterone suppresses apoptotic DNA fragmentation, testosterone was injected after castration. Treatment

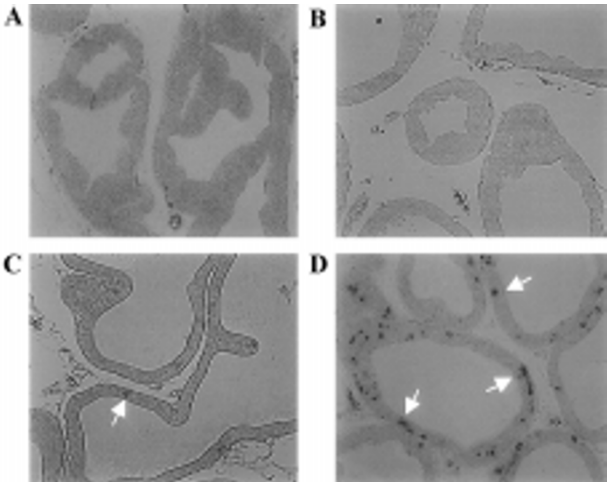

Figure 3. TUNEL assay in the ventral prostate following castration. The TUNEL method was used to detect apoptotic cells *in situ* in formalin-fixed, paraffin embedded sections of ventral prostate tissue. Results from an assay including ventral prostate tissue from sham-operated rat (A), 6 h (B), 24 h (C) and 48 h (D) after castration are shown. The other assays were performed as described in 'Materials and Methods'. Magnification × 200.

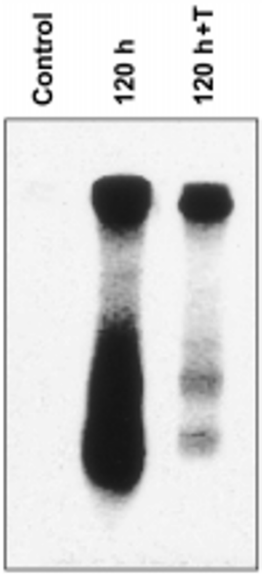

Figure 4. Effect of testosterone on castration-induced DNA fragmentation in the ventral prostate. Testosterone propionate (5 mg) was administered immediately after castration. The other assays were performed as described in Figure 2.

with testosterone almost blocked castration-induced apoptotic DNA cleavage (Figure 4). The result suggests that the castration-dependent apoptotic DNA cleavage is likely caused by testosterone withdrawal.

Castration-dependent early induction of *c-myc* mRNA is correlated with DNA synthesis in the ventral prostate

It is known that the expression of *c-myc* is enhanced during the androgen-induced proliferation and regrowth of the involuted prostate (Furuya and Isaacs, 1993). To investigate a biological function of an early induction of *c-myc* mRNA after castration, DNA synthesis was determined using [^{3}H]thymidine. After castration, DNA synthesis rapidly increased and then reached to a peak at 6 h. Subsequently, the DNA synthesis gradually decreased (Figure 5).

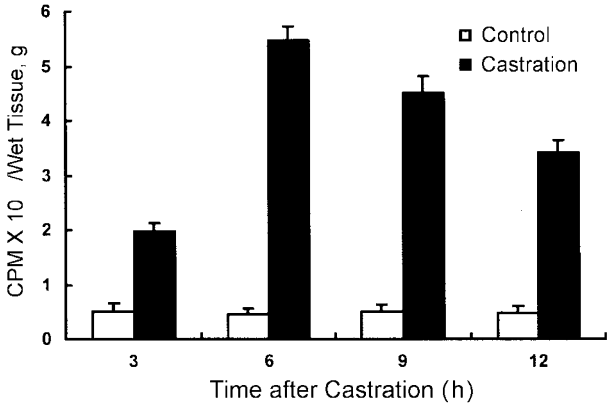

Figure 5. Incorporation of radiolabeled thymidine into the ventral prostate after castration. Rats were intraperitoneally injected with ^{3}Hthymidine (20 μCi/100 g of body weigth in 0.4 ml of sterile saline) and pulsed for 2 h. Rats were killed at each time indicated in the Figure. The total uptake expressed as count per minute (× 10^4) per g of the ventral prostate is shown. The other assays were performed as described in Materials and Methods. The results represent the mean of triplicate samples from three separate experiments.

Discussion

Apoptosis is a gene-directed process and can be seen, along with more familiar gene-directed process like differentiation, as a part of the repertoire available to the cellular response to external and internal stimuli. c-Myc is a very important element in cell proliferation, but it is continuously present under conditions of growth arrest, such as growth factor deprivation, which can induce apoptosis (Bissonnette *et al.*, 1994). In addition, *c-myc* antisense oligonucleotides inhibit the activation-induced cell death of T cell hybridoma (Shi *et al.*, 1992), suggesting that expression of endogenous *c-myc* is required for an induction of apoptosis in this situation. c-Myc can therefore induce both proliferation and apoptosis, and the cellular decision between these two responses is determined by other signals, such as the presence of growth factors or other survival stimuli. Androgen ablation-induced prostatic cell death involves a genetically programmed series of events, which are initiated when prostatic androgen decreases beyond a critical point (Fruya and Isaacs, 1993). c-Myc mRNA is also increased during prostatic glandular cell proliferation by an

androgen treatment after castration and gradually decreased. In our experiments, *c-myc* mRNA was differentially induced and reached a maximum level at 6 hours and 48 h, respectively. Therefore castration-dependent early induction of *c-myc* mRNA may be related to a partial decrease of dihydrotestosterone concentration in the ventral prostate after castration.

Apoptotic cells are infrequently observed in the ventral prostate of intact rat. There is no change in the percentage of apoptotic epithelial cells, whereas apoptotic bodies are strikingly apparent on day 2 following castration (English *et al*., 1989). On the other hand, Nickerson *et al.* reported that apoptosis in the ventral prostate, as detected in situ by the TUNEL method, was also induced as early as 6 h after castration (Nickerson *et al*., 1998). In the present data, apoptotic body and DNA fragmentation were not entirely detected at 6 h after castration. The result was quite different from the report of Nickerson *et al*., which apoptotic DNA fragmentation and apoptotic body were detected at 6 h in the ventral prostate after castration and they were exhibited at 24 h and rapidly increased at 48 h (1998). Therefore the apoptosis of the ventral prostate is closely correlated only with a late *c-myc* induction and is androgen-dependent. c-Myc is induced by a two-way state in cells that the cell chooses either apoptosis or proliferation, depending on the presence or absence of second "survival" signals such as growth factors (Bissonnette *et al*., 1994). However, an early induction of *c-myc* mRNA in the ventral prostate after castration is not related to apoptosis. In summary, protooncogene *c-myc* is differentially regulated in the ventral prostate after castration, and an early induction of *c-myc* mRNA is related to proliferation of the ventral prostate, whereas a late induction is related to apoptotic DNA fragmentation. The physiological significance of an early induction of *c-myc* mRNA in the ventral prostate after castration is not known as yet.

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References

Barbiroli, B. and Porter, V. R. (1971) DNA synthesis and interaction between controlled feeding schedules and partial hepatectomy in rats. *Science* 172: 738-741

Bissonnette, R. P., Shi, Y., Mahboubi, J. M. and Green, D. R. (1994) c-Myc and apoptosis. In *Apoptosis II* (Tomei, L.D., and Cope, R.O., eds), pp327-356, CSHL Press, New York

Bruchovsky, N. and Wilson, J. D. (1968) The intranuclear binding of testosterone and 5α-androstan-17β-ol-3-one by rat prostate. *J. Biol. Chem.* 243: 5953

Buttyan, R., Zakeri, Z., Lockshin, R. and Wolgemuth, P. (1988) Cascade induction of c-fos, *c-myc*, and heat shock 70 k transcripts during regression of the rat ventral prostate gland. *Mol. Endocrinol*. 2: 650-657

Cha, S. C., Suh, K. S., Song, K. S. and Lim, K. (2000) Cell death in retinoblastoma: Electron microscopic, immunohistochemical, and DNA fragmentation studies. *Ultrastruct. Pathol.* 24: 23-32

Colombel, M., Olsson, C. A., Ng, P. Y. and Buttyan, R. (1992) Hormone-regulated apoptosis results from reentry of differentiated prostate cells onto a defective cell cycle. *Cancer Res.* 52: 4313-4319

English, H. F., Kyprianou, N. and Isaacs, J. T. (1989) Relationship between DNA fragmentation and apoptosis in the programmed cell death in the rat prostate following castration. *Prostate* 15: 233-250

Furuya, Y. and Isaacs, J. Y. (1993) Differential gene regulation during programmed death (apoptosis) versus proliferation of prostatic glandular cells induced by androgen manipulation. *Endocrinology* 133: 2660-2666

Isaacs, J. T. (1984) Antagonistic effect of androgen on prostatic cell death. *Prostate* 5: 545-557

Karlinsey, J., Stamatoyannopoulus, G. and Envr, T. (1989) Simultaneous purification of DNA and RNA from small number of eukaryotic cells. *Anal. Biochem*. 180: 303-306

Kelly, K., Cochran, B., Stiles, C. D. and Leder, P. (1983) Cell specific regulation of the *c-myc* gene by lymphocyte mitogens and platelet derived growth factor. *Cell* 35: 303-610

Kerr, J. F. R. and Searle, J. (1973) Deletion of cells by apoptosis during castration-induced involution of the rat prostate. *Virchows Arch. [Cell Pathol.]* 13: 87-102

Kyprianou, N. and Isaacs, J. T. (1988) Activation of programmed cell death in the rat ventral prostate after castration. *Endocrinology* 122: 552-562

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Land, H., Parada, L. F. and Weinber, R. A. (1983) Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. *Nature* 304: 596-602

Lee, J. H., Sul, C. K., Kim, Y. K., Hwang, B. D. and Lim, K. (1999) Differential regulation of protooncogene *c-myc* expression in rat ventral prostate after castration. *Biochem. Mol. Biol. Int.* 47: 143-151

Lim, K., Park, H. C., Kim, K. Y., Lee, M. S., Kweon, G. R., Kwak, S. T. and Hwang, B. D. (1993) Regenerating liver-specific transacting factors of H2B histone gene are newly synthesized during liver regeneration. *Biochem. Biophys. Res. Comm*. 196: 1093-1100

Lim, K., Yoo, J. H., Kim, K. Y., Kweon, G. R., Kwak, S. T. and Hwang, B. D. (1994) Testosterone regulation of proto-oncogene *c-myc* expression in primary Sertoli cell cultures from prepubertal rats. *J. Androl*. 15: 543-550

Makino, R., Hayashi, K. and Sugimura, T. (1984) *C-myc* transcript is induced in rat liver at a very early stage of regeneration or by cycloheximide treatment. *Nature* 310: 697-699

Nickerson, T., Pollak, M. and Huynh, H. (1998) Castration-induced apoptosis in the rat ventral prostate is associated with increased expression of genes encoding insulin-like growth factor binding proteins 2, 3, 4 and 5. *Endocrinology* 139: 807-810

Quamby, V. E., Beckmann, W. C., Wilson, E. M. and French, F. S. (1987) Androgen regulation of *c-myc* messenger ribonucleic acid levels in rat ventral prostate. *Mol. Endocrinology* 1: 864-874

Sandford, N. L., Searle, J. W. and Kerr, J. F. K. (1984) Successive waves of apoptosis in the rat prostate after repeated withdrawal of testosterone stimulation. *Pathology* 16: 406-410

Shi, Y. Glynn, J. M., Guilbert, L. J. H., Cotter, T. G., Bisonette, R. P. and Green D. R. (1992) Role for *c-myc* in activation-induced apoptotic cell death in T-cell hybridoma. *Science* 257: 212-214

Tilly, J. L. and Hsueh, A. J. W. (1993) Microscale autoradiographic method for the qualitative and quantitative analysis of apoptotic DNA fragmentation. *J. Cell Physiol*. 154: 519-526

Virca, G. D., Norhemann, W., Shiels, B. R., Widera, G. and Broome, S. (1990) Simplified northern blot hybridization using 5% sodium dodecyl sulfate. *BioTechniques* 8: 370-372