Effects of EGF and TGFβ_1 on Regulation of Proliferation and Expression of Immediate Early Genes in Embryonic Hamster Palate Mesenchymal Cells

By

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ABSTRACT

The study was undertaken to investigate: (1) the effects of serum and the growth factors, EGF and TGFβ_1, and their combination, on the proliferation of embryonic hamster palate mesenchymal cells (HPMC); and (2) the effects of serum, growth factors and their combinations on expression of the immediate early genes, *c-fos*, *c-jun*, and *c-myc*.

Initially, growth behavior of the cultured HPMC was examined. The results showed that the proliferation of HPMC was dependent on the concentration of serum in the culture media; and at least 2.5% serum was necessary to sustain the growth of HPMC in culture. EGF supported DNA synthesis (only in the presence of serum), and exerted mitogenic effects on HPMC; whereas TGFβ_1 did not support DNA synthesis and arrested growth of HPMC. In addition, following co-treatment of HPMC with EGF and TGFβ_1, the mitogenic effect on HPMC of EGF was inhibited by TGFβ_1. Also, 30 minutes of TGFβ_1 pre-treatment was sufficient to irreversibly inhibit the serum- and/or EGF-induced DNA synthesis as well as proliferation of HPMC.

Northern blot analysis showed that both serum and EGF induced rapid expression of *c-fos*, *c-jun*, and *c-myc*; whereas TGFβ_1 did not. Also, following co- or pre-treatment with TGFβ_1, the serum- and/or EGF-induced expression of immediate early genes was not inhibited. However, co- or pre-treatment with TGFβ_1 did result in modulations in the temporal expression pattern of these immediate early genes.

The results of the present study indicate that EGF and TGFβ_1 are important regulators of embryonic HPMC proliferation. Further, this study suggests that interaction among extracellular factors leads to modulation of the nuclear events that may be important in regulation of HPMC proliferation during palate morphogenesis.

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LIST OF ABBREVIATIONS

AP-1 - activator protein 1
ATP - Adenosine triphosphate
bFGF - basic fibroblast growth factor
cAMP - cyclic adenosine phosphate
cGMP - cyclic guanine monophosphate
CK2 - Casein Kinase 2
CM - complete media
CMF/PBS - calcium magnesium free/ phosphate buffered saline
Con A - Concanavalin A
CRE - cAMP response element
CREB - CRE binding protein
CSF - Colony stimulating factor
CTP - Cytosine triphosphate
DAG - diacylglycerol
DEPC - diethylpyrocarbonate
DMEM - Dulbecco's Modified Eagle Medium
DNAPK - DNA protein kinase
ECM - Extracellular matrix
EDTA - Ethylene diamine tetra acetic acid
EGF - Epidermal growth factor
EGFR - EGF receptor
FCS - Fetal calf serum
FGF - Fibroblast growth serum
FKBP - FK506 binding protein (a peptidyl polyisomersase)
FT-α - farnesyltransferase
GAG - Glycosaminoglycan
GAL4 - positive regulator of galactokinase gene
GAPDH - glyceraldehyde 3-phosphate dehydrogenase
GSK - glycogen synthase kinase
GTP - Guanosine triphosphate
HPMC - Hamster palate mesenchymal cells
IGF - Insulin growth factor
IGFBP - IGF binding protein
IL - interleukine
JAK - Janus kinase
JNK - c-jun N-terminal kinase
MAD - mothers against dpp
MAPK - Mitogen-activated protein kinase
MEE - Medial edge epithelium
MEK - MAPK kinase
MEL - Myeloid erythroleukemia
NGF - Nerve growth factor
NLS - nuclear localizing signal
PDGF - Platelet-derived growth factor
PGs - Prostaglandins
PI - phosphatidyl inositol

PIP2 - phosphatidyl inositol bis-phosphate
PLA2 - Phospholipase A2
PLC - Phospho-lipase C
PKA - Protein kinase A (cAMP dependent protein kinase)
PKC - Protein kinase C
PMA - Phorbol myristate acetate
Rb - retinoblastoma protein
RSRF - related to serum response factor
SAPK - stress activated protein kinase
SHC - Src homology/collagen
SIE - serum inducible element
SRE - serum response element
SRF - serum response factor
STAT - signal transducers and activators of transcription
TAK1 - TGFß -activated kinase
TßR - TGFß receptor
TCF - ternary complex factor
TGFα - Transforming growth factor α
TGFß - Transforming growth factor ß
TNFα - tumor necrosis factor α
TPA - 12-O- tetradecanoylphorbol 13 acetate
TRE - TPA response element

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INTRODUCTION

Morphogenesis of Mammalian Palate

Morphogenesis of mammalian palate is sequential and unique. It starts as intraoral outgrowth of two bilaterally symmetrical projections (shelves) from the maxillary processes in a vertical direction toward the floor of the mouth. The vertical shelves then reorient to a horizontal plane, and finally unite with one another in the midline, resulting in the separation of the oral and nasal cavities (Greene and Pratt, 1976; Shah, 1984).

The sequential events of secondary palate morphogenesis, i.e., vertical growth, reorientation, and fusion of the palatal shelves are peculiar to mammals. In other vertebrates, formation of the secondary palate is relatively simple. For instance, in early vertebrates, such as fish and amphibia, the palatal shelves grow vertically from the maxillary processes. The palate morphogenesis, however, does not advance and the shelves remain vertical throughout the ontogeny of the organism (LeCluyse et al., 1985; Shah et al., 1990). In the alligator, the only reptile studied so far, the palatal shelves grow, *ad initium*, from the maxillary processes in a horizontal direction and unite, thus separating the oral and nasal cavities (Ferguson, 1981). In birds, as in reptiles, the palatal shelves start out in a horizontal direction towards each other but they never unite, and a physiological cleft persists throughout avian ontogeny (Shah and Crawford, 1980; Koch and Smiley, 1981; Shah et al., 1985a, 1987, 1988).

A further review of the literature on palate development shows that mammals have been the target of most studies concerning the mechanisms that regulate morphogenesis of the secondary palate in vertebrates. These studies indicate that each step of palate morphogenesis involves a number of complex cellular and molecular events.

Initially, for the formation a vertical palate primordia, cell proliferation seems to be a major contributor to the shelf growth (Shah et al., 1989a, b, c; 1994b). For further

progression of vertical shelf morphogenesis, synthesis of extracellular matrix (ECM) molecules such as glycosaminoglycans (GAGs), various collagens, and fibronectin (Pratt and King, 1971; Silver et al., 1981; Benkhaial and Shah, 1994; Young et al., 1994a) appears to be necessary to regulate the size and shape of the palatal shelves.

Subsequently, spatio-temporally regulated synthesis and accumulation of ECM molecules such as sulfated and non-sulfated GAGs, collagens, and fibronectin have been suggested to play a significant role in the reorientation of the palatal shelves from a vertical to a horizontal plane (Larsson, 1962; Jacobs, 1964; Nanda, 1971; Pratt et al., 1973; Ferguson, 1978; Brinkley, 1980; Jacobson and Shah, 1981; Brinkley and Vickerman, 1982; Brinkley and Morris-Wiman, 1984; Turley et al., 1985; Foreman et al., 1991; Benkhaial and Shah, 1994; Singh et al., 1994; 1997; Young et al., 1994a; Ohsaki et al., 1995). It has been proposed that regional accumulation and the increased synthesis of various matrix molecules during reorientation of the palatal shelves may create an environment within the palatal shelves to facilitate the migration of palate mesenchymal cells (Lassard et al., 1974; Krawczyk and Gillon, 1976; Wee and Zimmerman, 1980; Shah, 1979b; Brinkley, 1980; Venkatasubramanian and Zimmerman, 1983), which, in turn, would cause the shelves to reorient.

Following reorientation, the palatal shelves approximate and contact one another. Prior to the contact of the two opposing shelves, the medial edge epithelial (MEE) cells cease DNA synthesis (Hudson and Shapiro, 1973; Pratt and Martin, 1975; Shah et al., 1985b), accumulate lysosomal enzymes (Hayward, 1969; Smiley, 1970; Shah and Chaudhry, 1974; Im and Muliken, 1983; Shah et al., 1991) and increase cyclic AMP levels (Pratt and Martin, 1975; Greene and Pratt, 1979; Greene et al., 1980; Shah et al., 1985b). Subsequently, the MEE of the opposing palatal shelves adhere to each other by means of a surface glycoprotein coat (Greene and Kochhar, 1974; Pratt and Hassell, 1975; Greene and

Pratt, 1977; Heinen et al., 1982; Baeckeland et al., 1982) and desmosomes (Shah, 1979) to form an epithelial seam. The epithelial cells of the seam then disappear and mesenchymal continuity is established between the united palatal shelves. Several studies have suggested that programmed cell death may account for the elimination of the MEE cells from the midline seam (Mato et al., 1966; Smiley, 1970; Chaudhry and Shah, 1973; Schupbach and Schroeder, 1983; Mori et al., 1994; Taniguchi et al., 1995). The programmed cell death may be regulated by epithelial-mesenchymal interactions (Shah, 1984; Ferguson et al., 1984). On the other hand, it has been suggested that the epithelial cells of the midline seam, rather than being eliminated by cell death, may be transformed into mesenchymal cells (Fitchett and Hay, 1989; Griffith and Hay, 1992; Shuler, 1995; Yano et al., 1996a). Recently, Carette and Ferguson (1992) corroborated an earlier proposal made by Chaudhry and Shah (1973) that some of the midline epithelial cells may migrate to and integrate with or be eliminated from the adjacent oral and nasal epithelia to facilitate mesenchymal union.

Regulation of Palate Morphogenesis

Recent studies have suggested that the cellular and molecular events that occur during growth and differentiation of embryonic palate morphogenesis may be regulated by growth factors, prostaglandins, and neurotransmitters.

Using immunohistochemical, Western and Northern blots, as well as *in situ* techniques, many growth factors and/or their receptors have been localized in the developing palate of mammals (Table 1), implicating their involvement during palate morphogenesis.

Much of the work on growth factor involvement in regulation of embryonic palate development has been carried out using epidermal growth factor (EGF) and transforming growth factorβ_1 (TGFβ_1). EGF was the first growth factor studied in palate development

(Hassell, 1975; Hassell and Pratt, 1977). Since then, EGF/TGFα and their receptor molecules have been localized in both mesenchymal and epithelial cells during all stages of palate formation (Abbott et al., 1988; Abbott and Birnbaum, 1990; Shiota et al., 1990; Dixon et al., 1991; Brunet et al., 1993; Citterio and Gaillard, 1994; Jaskoll et al., 1996). Furthermore, several tissue and cell culture studies using palatal cells have shown that both palatal mesenchymal and epithelial cells respond to EGF or TGFα (Nexo and Pratt, 1980; Pratt, 1980; Pratt et al., 1984; Silver et al., 1984; Greene and Lloyd., 1985; Turley et al., 1985; Pratt, 1987; Gawel-Thompson and Greene, 1989; Dixon and Ferguson, 1992; Chepenik et al., 1994; Shah et al., 1995). Exogenous EGF appears to prevent programmed cell death of the MEE and result in its differentiation towards a keratinization epithelium (Hassell and Pratt, 1977; Pratt, 1980; Dixon and Ferguson, 1992; Brunet et al., 1993). Both the palate epithelial and mesenchymal cells in culture have also been shown to proliferate and produce ECM molecules in the presence of EGF/TGFα (Gawel-Thompson and Greene, 1989; Foreman et al., 1991; Sharpe et al., 1992a,b; Dixon et al., 1993a,b; Shah et al., 1995). These findings suggest that EGF/TGFα may influence both the proliferation and differentiation of palatal cells, and thus contribute to the formation of the palatal shelves.

Several studies have investigated spatio-temporal localization of $TGF\beta_1$ molecules in both the epithelial and mesenchymal cells at various stages of palate development (Table 1) (Heine et al., 1987; Pelton et al., 1990a,b; Fitzpatrick et al., 1990; Abbott and Birnbaum, 1990; Gehris et al., 1991; Williams et al., 1991; Proetzel et al., 1995; Jaskoll et al., 1996). Treatment of cultured embryonic palate with $TGF\beta_1$ results in precocious cessation of DNA synthesis in the MEE, and accelerated palatal fusion (Shuler et al., 1991, 1992; Gehris and Greene, 1992). Exogenous $TGF\beta_1$ inhibits proliferation (Linask et al., 1991; Sharpe et al., 1992a,b), enhances GAG production, and increases synthesis but decreases

degradation of collagen in palate mesenchymal cells (D'Angelo and Greene, 1991). Recent studies also suggested that TGFβ_3 may be necessary for normal closure of palatal shelves: palatal shelf fusion is blocked by TGFβ_3 antisense oligomers or neutralizing antibodies (Shuler et al., 1991; 1992; Gehris and Greene, 1992; Proetzel et al., 1995). The mechanisms by which TGFβs affect palatal cell behavior is, however, not yet understood.

Sharpe and associates (1992a) have indicated that treatment of palate mesenchymal cells with TGFβ_1 or IGF II induces reduction of EGF receptor binding. The effects of bFGF on EGF receptor-ligand binding kinetics seems to be biphasic: a short period of treatment (3-5 hours) induces a decrease, but a long period of treatment (24 hours) results in a large increase in ^{125}I-EGF binding. Co-incubation of TGFβ_1 with bFGF inhibits the positive effects of bFGF on EGF receptor binding. Further, pre-treatment of palate mesenchymal cells with either bFGF or IGF II enhances the ^{3}H-thymidine incorporation induced by EGF treatment; whereas it is reduced with TGFβ_1 (Sharpe et al., 1992a). Simultaneous treatment with TGFβ_1 and PDGF stimulates ^{3}H-proline incorporation in palate mesenchymal cells (Sharpe et al., 1992b). Also, regulation of TGFβ_3 expression in murine embryonic palatal cells appears to be upregulated by treatment with TGFβ_1 and β_2, but downregulated by EGF treatment (Gehris et al., 1994).

Table 1. Growth factors and/or receptors during mammalian palate development.

Growth factor	Location in palatal tissue	Stage of palate development	Authors
EGF (protein)	M and E	all stages	Abbott and Birnbaum, 1990; Dixon et al., 1991
EGF/TGFα receptor (protein)	M and E	all stages	Abbott et al., 1988; Shiota et al., 1990; Dixon et al., 1991; Citterio and Gaillard, 1994; Jaskoll et al., 1996
TGFα (protein)	M and E	all stages	Abbott and Birbnaum, 1990; Dixon et al., 1991; Citterio and Gaillard, 1994
FGF acidic and basic (protein)	M and E	fusion	Sharpe et al., 1993
TGFβ1 (protein)	M and E	vertical and horizontal	Heine et al., 1987; Abbott and Birnbaum, 1990; Williams et al., 1991; Gehris et al., 1991
TGFβ2 (protein)	M and E	all stages	Abbott and Birnbaum, 1990; Gehris et al., 1991
TGFβ1 (mRNA)	M and E	vertical and horizontal	Fitzpatrick et al., 1990; Pelton et al., 1990; Jaskoll et al., 1996
TGFβ2 (mRNA)	M and small regions of MEE	vertical and horizontal shelf, and during fusion	Fitzpatrick et al., 1990; Jaskoll et al., 1996
TGFβ3 (mRNA)	MEE	vertical and horizontal	Fitzpatrick et al., 1990; Jaskoll et al., 1996
TGFβ receptors (types I, II, & III) (protein)	M	vertical (cell culture)	Linask et al., 1991
PDGF-AA (protein)	basement membrane, M nasal E, and MEE	all stages	Qui and Ferguson, 1995
PDGF-BB (protein)	E	horizontal	Qui and Ferguson, 1995
PDGF-α receptor (protein)	M, E; heavy in MEE	vertical and horizontal	Qui and Ferguson, 1995
PDGF-β receptor (protein)	nasal E	post-fusion	Qui and Ferguson, 1995
IGF I (protein)	M	vertical and horizontal	Ferguson et al., 1992
IGF II (protein)	E	vertical and horizontal	Ferguson et al., 1992
IGF II (mRNA)	M	horizontal	Ferguson et al., 1992
IGFBP-1(protein)	E	horizontal	Ferguson et al., 1992

M: mesenchyme
E: epithelium
MEE: medial edge epithelium

In addition to growth factors, prostaglandins (PG) have been implicated in the regulation of mammalian palate development (Greene and Garbarino, 1984). Various prostaglandins such as PGE_2 and PGF_2, and their receptors have been immunolocalized in developing palatal tissue (Greene and Lloyd, 1985; Jones and Greene, 1986), and indeed are synthesized by palate mesenchymal cells (Chepenik and Greene, 1981; Alam et al., 1982). Experimental evidence suggested that PGE_2 and PGI_2 induce cAMP synthesis in primary cultures of palate mesenchymal cells (Greene et al., 1981b), stimulate GAG synthesis (Greene et al., 1982), and inhibit re-entry of cells into the cell cycle (Greene et al., 1981a, b; Pisano et al., 1986). Elevation of intracellular levels of cAMP seem to partially inhibit the release of various prostaglandins (Chabot and Chepenik, 1986). These data suggest possible involvement of PGs in the regulation of proliferative and differentiative activities of palatal mesenchyme (Chabot and Chepenik, 1986).

Several neurotransmitters such as dopamine, norepinephrine, and epinephrine, as well as β-adrenergic receptors have been detected in the developing mammalian palate (Zimmerman et al., 1981; Zimmerman and Wee, 1984; Pisano et al., 1986; Pisano and Greene, 1987; Greene, 1989). Exposure of palatal cells to neurotransmitters activates β-adrenergic receptors, which leads to stimulation of adenylate cyclase activity and subsequent accumulation of intracellular cAMP in a dose-dependent manner (Waterman et al., 1976, 1977; Garbarino and Greene, 1984; Greene and Garbarino, 1984). Also, addition of isoproterenol, a potent β-agonist, increases cAMP levels in palatal cells and delays re-entry of cells into the cell cycle (Pisano et al., 1986; Greene, 1983). Furthermore, the neurotransmitters, seratonin and acetylcholine, appear to stimulate palatal shelf reorientation, whereas γ-amino-n-butyric acid (GABA) inhibits it (Zimmerman and Wee, 1984). These data suggest putative involvement of these neurotransmitters in the regulation of palate morphogenesis.

The Foregoing analysis suggests that extracellular factors are essential regulators of cellular functions during palate morphogenesis. To understand the mechanisms by which growth factors, prostaglandins, and neurotransmitters regulate growth, proliferation, and differentiation of mammalian embryonic palate mesenchymal cells, recent studies have investigated the involvement of intracellular signaling molecules (figure 1). It has been suggested that palate mesenchymal cell behavior may be regulated by several intracellular signaling cascades, and cross-communication among them, which are involved in relaying the extracellular signals from the plasma-membrane to the nuclear environment. For example, exposure of vertebrate palate mesenchymal cells to extracellular factors seems to affect the cellular levels of cAMP, which in turn, modulates the activity of protein kinase A (PKA) and subsequently induces changes in cell cycle progression and extra-cellular matrix synthesis (Greene et al., 1982; Pisano et al., 1986; Pisano and Greene, 1986). Also, treatment of palate mesenchymal cells with growth factors induces the activation of PKC (Chepenik and Grunwald, 1988; Chepenik and Haystead, 1989), and that of second-messenger independent protein kinases such as mitogen activated protein kinase (MAPK) , casein kinase 2 (CK2), and $p34^{cdc2}$ (Young et al., 1995, 1996a, b), all of which have been implicated in regulation of cellular behaviors including cell proliferation and differentiation. Although several extracellular ligand-regulated signaling cascades in palate mesenchymal cells have been recognized (figure 1), the information on how the signaling molecules mediate the down stream nuclear events in response to different factors is not available. Recently Greene and associates (1995) have identified an increase in the activity of CRE binding protein (CREB) with advancing palate development. CREB is a transcription factor that binds to the promoter regions of several genes and seems to mediate the linkage between cAMP and gene expression. In fact, *in vitro* induction of cAMP in palate

mesenchymal cells has also been shown to result in an increase of CREB phosphorylation, and hence an increase in its activity.

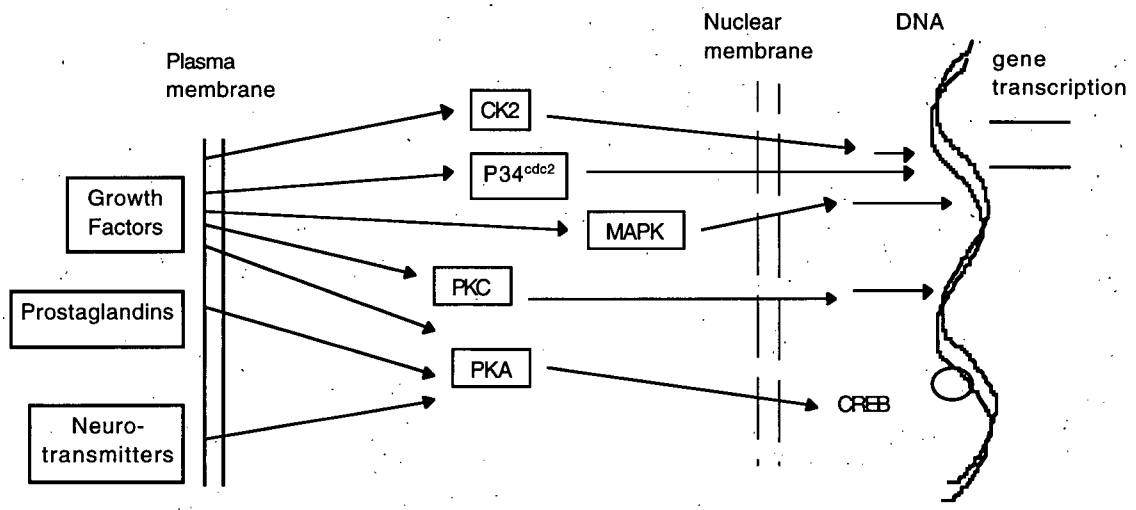


Figure 1. Schematic presentation of current state of understanding of regulation of growth and differentiation of mammalian embryonic palate mesenchymal cells.

Epidermal Growth Factor (EGF)

EGF is one of the best known growth factors. It was first isolated from the submaxillary gland of mice by Cohen in 1960, and was shown to cause premature opening of eyelid and eruption of teeth in neonatal mice (Cohen, 1962). During the past three decades, EGF related molecules were identified in many eukaryotes and were shown to play critical roles during development, repair, and maintenance of a variety of tissues in different organisms (Cochet, 1989; Carpenter and Wahl, 1990). In addition, EGF seems to exert mitogenic effects in tissues of endodermal or mesodermal origins, and thus participate in regulation of proliferation in these tissues (Hofmann and Scott, 1995).

EGF is a single poly-peptide chain of 53 amino acids, containing 3 intramolecular disulfide bonds, which are required for its biological activity (Taylor et al., 1972; Savage et al., 1973). Precursor EGF is first expressed as a large 1200 amino acid long, glycosylated, membrane anchored molecule (Bell et al., 1986; Mroczkowski et al., 1989), which is processed in a tissue specific manner to soluble EGF molecule (Cochet, 1989). Besides the active domain, the precursor EGF molecule contains 8 EGF-like sequences (a set of 6 cysteine residues spaced over a span of 30-40 amino acids), and a low density lipoprotein (LDL) receptor-like domain (Bell et al., 1986). In addition to the unbound EGF molecule, the high molecular weight membrane-bound precursor EGF molecule seems to be biologically active and has been suggested to play a role in cell-cell recognition (Mroczkowski et al., 1989).

EGF receptor

The EGF receptor is a 170 kDa transmembrane glycoprotein, which belongs to the tyrosine kinase family of receptors (Carpenter and Zendegui, 1986; Gill et al.,1987). The EGF receptor consists of an extracellular ligand-binding domain, a trans-membrane region, and a cytoplasmic domain, which contains a juxta-membrane region, a catalytic domain, and a C-terminal tail with at least five tyrosine phosphorylation sites (Carraway and Cantley, 1994; Carter and Kung, 1994; Boonstra et al., 1995). The intracellular region of the EGF receptor contains a number of tyrosine, and serine/threonine phosphorylation sites that seem to play important regulatory roles in activation of the receptor (Ullrich et al., 1984; Downward et al., 1984; Davis and Czech, 1985; Hunter et al., 1985; Carpenter and Wahl, 1990; Staros and Guyer, 1995). EGF can bind with both low and high affinity to the extracellular domain of the EGF receptor (Livneh et al., 1986). The binding of EGF to the receptor on the cell membrane results in oligomerization and autophosphorylation of the

receptor, which leads to activation of a number of intracellular protein substrates, and subsequently to the activation of various signal transduction cascades in the cell (Carter and Kung, 1994; Boonstra et al., 1995). These signal transduction cascades then form a biochemical network, which ultimately induces metabolic alterations and intrinsic molecular changes in gene expression that modulate the cells' behavior (Cochet, 1989; Ullrich and Schlessinger, 1990; Carpenter and Wahl, 1990). Also, upon ligand binding to the receptor, the EGF-receptor complex is internalized, stored in intracellular compartments, and is eventually degraded in lysosomes (Carpenter and Cohen, 1976; Stoschek and Carpenter, 1984; Beguinot et al., 1984; Schlessinger, 1986; Cochet, 1989).

EGF signaling pathways

Following the ligand binding, autophosphorylation of the cytoplasmic domain of the EGF receptor is a critical step in initiation of various signaling pathways. The phosphorylated regions form binding sites for different cytoplasmic proteins that mediate several signaling pathways. The proteins that directly interact with the phosphorylated cytoplasmic domain of EGF receptor include enzymes such as phospholipase Cγ (PLCγ), Raf, Ras-GTPase activating protein (Ras-GAP), *syp* phosphotyrosine phosphatase, and non-enzymatic molecules such as p85 subunit of phosphatidylinositol 3-kinase (PI3 kinase), Src homology/collagen (SHC), growth factor receptor-bound protein-2 (GRB2), and transcription factor p91 (Panayotou and Waterfield, 1993; Koch et al., 1994; Malarkey et al.,1995). These molecules are involved in induction of a number of signaling cascades, including second-messenger dependent cascades, mitogen-activated protein (MAP) kinase cascade, and the signal transducers and activators of transcription (STAT) cascade.

Second-messenger dependent cascades are among the signaling pathways involved in relaying EGF signal from the cell membrane to the intracellular environment. EGF receptor

activation leads to activation of PLCγ, which catalyzes phosphatidylinositol-4,5-bisphosphate (PIP2) to triphosphate inositol (PI3) and diacylglycerol (DAG) (Rhee et al., 1989). Accumulation of PI3 increases the intracellular concentration of Ca^{2+}, which together with DAG induce the activation of protein kinase C (PKC) (Nishizuka, 1988; Asaoka et al., 1992; Berridge, 1993). PKC has a broad range of substrates including growth factor receptors, ion channels, cytoskeletal proteins, nuclear proteins, several proto-oncogenes, as well as members of other signaling pathways such as MAP kinases (Pelech et al., 1990; Nishizuka, 1992; Olson et al., 1993; Mahoney and Huang, 1994; Hii et al., 1995).

In addition, involvement of second-messenger dependent heteromeric G-proteins in EGF signaling pathways has been proposed (Ramirez et al., 1995). For example, G-protein subunits seem to be involved in regulation of Ca^{2+} influx upon EGF signaling (Maraca, 1986; Moolenaar et al., 1986). Furthermore, transient association and activation of Giα subunit of G proteins with the ligand activated EGF receptor have been reported (Yang et al.,1991). G-proteins have also been suggested to play a role in EGF-induced activation of adenylate cyclase (Nair et al., 1990), resulting in an increase in cAMP levels in a number of cell types (Nair et al., 1990; Yu et al., 1992; Nakaguwa, 1991). The exact role and mechanism of action of G-proteins and cAMP in EGF-induced signal transduction pathways, however, remains unknown.

The best known EGF induced signal transduction pathway is the MAP kinase cascade (Boonstra et al., 1995; Carraway and Carraway, 1995). To initiate the intracellular pathway, the adaptor protein, GRB-2 binds to the phosphorylated EGF receptor and recruits a Ras guanine nucleotide exchange factor (Lowenstein et al., 1992; Boguski and McCormick, 1993; Panayotou and Waterfield, 1993), which interacts directly with Ras and induces the GDP/GTP exchange resulting in activation of Ras (Chardin et al., 1993; Gale et al., 1993). Activated Ras protein acts as a key mediator between the tyrosine receptor and the

proceeding intracellular protein kinases which include: Raf, MAP kinase kinase (MEK), and MAPK (Davis, 1993; Katz and McCormick, 1997). There are multiple kinase isoforms at each junction-point that allow the formation of a network of interactions for cross-communication between different signaling pathways. The progressive phosphorylation and activation of these protein kinases lead to the selective phosphorylation of various cytoplasmic and nuclear substrates such as S6K, p90rsk, c-Raf, c-Jun, c-Myc, and ELK-1 (Hunter and Karin, 1992; Fu and Zhang, 1993; Kazalauskas, 1994; Gupta et al., 1996) which ultimately affect the gene transcription and subsequent proliferative or differentiative behavior of the cell.

Recently, another group of substrates have been shown to interact with phosphorylated EGF receptors, which include STAT proteins (Sadowski, 1993; Zhong et al., 1994, Kumar et al., 1995; Leaman, et al., 1996). Activation of the EGF receptor results in rapid phosphorylation of these molecules (Sadowski, 1993; Darnell et al., 1994, David et al. 1996) and their translocation to the nucleus, where they act as transcription factors (Zhong et al., 1994; Leaman et al., 1996).

Transforming Growth Factor β (TGFβ)

Transforming growth factor β's (TGFβ) are a large family of well characterized peptide growth factors. Based on structural and functional properties, the members of the TGFβ superfamily of growth factors have been categorized into three subgroups: 1) TGFβ subfamily (mammalian TGFβ1-3, *Xenopus* TGFβ4, and chicken TGFβ5), 2) Activins/inhibins, and 3) Bone morphogenic proteins (BMP's, nodal, *Xenopus* Vg-1, *Drosophila* Dpp, and screw) (Massagué, 1990; Roberts and Sporn, 1990). In addition, other TGFβ related peptides, such as mullerian inhibiting substance (MIS) and glia derived

neurotrophic Factor (GDNF) have been identified, but they do not seem to belong to any of the categorized subfamilies (Massagué, 1990; Brand and Schnider, 1995; Polyak, 1996).

TGFβ1, the prototypical member of the TGFβ superfamily, was first isolated from human platelets by Assoian and associates in 1983, and later cloned from a human cDNA library by Derynck and co-workers in 1985. Subsequent studies have indicated that members of the TGFβ superfamily have a variety of functions during development, repair, and maintenance of tissues in evolutionary diverse organisms ranging from insects, worms, and frogs, to mammals (Massagué, 1990; Roberts and Sporn, 1990). Experiments on cultured cells, obtained from a variety of different organisms, suggest that these molecules may be involved in regulation of a wide spectrum of cellular behaviors such as cell proliferation, differentiation, migration, adhesion, ECM synthesis, and death (Lyons and Moses, 1990; Massagué, 1990; Roberts and Sporn, 1990; Kingsley, 1994).

Understanding the role of TGFβ molecules during mammalian development has been the subject of intense investigation during the past decade. Numerous studies have employed techniques such as Northern and Western blottings and *in situ* hybridization of mRNA as well as immuno-localization of protein to localize TGFβ molecules in developing mammalian systems (Heine et al., 1987; Wilcox and Derynick, 1988; Lenhert and Akhurst, 1988; Miller et al., 1989; Pelton et al., 1990a, b; 1991). Even though there is some disagreement among different studies regarding the exact onset and amount of TGFβ expression during mammalian development (Akhurst, 1994), these studies generally recognize the presence of both mRNA and protein of TGFβ in the developing tissues and organs such as haemopoetic tissue, salivary gland, tooth bud, secondary palate, eye, neural tissue, bone tissue, and kidney (Akhurst et al., 1992; Akhurst, 1994), thus implicating their involvement in embryonic development.

Structurally, TGFβ molecules are disulfide-linked dimers of two identical polypeptide chains, which contain nine conserved cysteine residues. These molecules are initially synthesized by a variety of cell types in a precursor form, composed of a N-terminal signal sequence, a pro-region containing glycosylation sites, and a C-terminal bioactive region (Massagué, 1990). Upon secretion to the extracellular environment, the pro-region cleaves, but remains non-covalently associated with the bioactive form. However, only the dissociated bioactive TGFβ molecule is capable of binding to the receptors and performing biological activities. The dissociation of bioactive TGFβ thus appears to be tightly regulated in various tissues or cell culture environments (Massagué, 1990).

TGFβ receptors (TβR)

At least five membrane-bound glycoprotein receptors of TGFβ have been identified: TβRI, II, III, IV, and V (Yingling et al., 1995; Kolodziejczyk and Hall, 1996). However, only TβR I, II, and III, which seem to be abundantly expressed in almost all cell types, have been well characterized (Cheifetz et al., 1987, 1990; Massagué,1987, 1990, 1992, Attisano et al., 1994; Kingsley, 1994). Both TβR I and TβR II contain cytoplasmic serine/threonine protein kinase domains, whereas TβR III is a membrane anchored proteoglycan, and lacks an intracellular kinase domain (Cheifetz et al., 1988; Massagué, 1990).

TheTβR I (50-60 kDa) belongs to the family of serine/threonine kinase receptors, which are ubiquitously expressed in various cell types of different species, and share 60-70% identity in their kinase domains. Studies on TGFβ resistant mutants have shown that TβR I is essential for mediating the TGFβ signal across the cytoplasmic membrane, although it is unable to bind to TGFβ directly (Boyd and Massagué, 1989; Laiho et al.,

1990). Instead, TβR I seems to recognize and interact with TGFβ-bound type II receptor (Wrana et al., 1992; Bassing et al.,1994).

The TβR II (75 kDa) also belongs to the family of serine/threonine kinase receptors, which has been identified in mammals, *Drosophila*, and *C. elegans* and show 30-40% homology in the kinase domain (Massagué et al., 1994). Studies on TGFβ non-responsive mutants have demonstrated that, like TβR I, TβR II is required for transducing TGFβ signals from extra- to intra-cellular environment (Laiho et al., 1990). However, unlike TβR I, TβR II seems to bind directly to TGFβ molecules (Laiho et al., 1990; Wrana et al., 1992).

TβR III (280-330 kDa), also known as betaglycan, is a large membrane-bound proteoglycan, which binds to all three isoforms of TGFβ but lacks a protein kinase domain (Massagué, 1985; Massagué and like, 1985; Cheifetz et al., 1987; Segarini and Seyedin, 1988; Lopez-Casillas et al., 1991; Fukushima et al., 1993). The TβR III may bind to TGFβ, and present TGFβ to its signaling receptors (type I and II), thereby increasing the signaling efficiency (Lopez-Casillas et al., 1993; Moustakas et al., 1993; Aittisano et al.,1994). Furthermore, in the presence of extracellular enzymes such as plasmin, TβR III becomes soluble and acts as an antagonist of TGFβ by preventing its receptor binding (Andres et al., 1989; Lammar et al., 1994; Lopez-Casillas et al., 1994). Thus, it seems that TβR III may play a dual role in regulating TGFβ activity: as a membrane-bound protein, it functions as an accessory molecule; and as a soluble molecule it acts as a sequestering molecule (Attisano et al., 1994).

The ligand binding and activation of TGFβ receptor complex have been elucidated in various cell types (Wrana et al.,1992; 1994; Lin and Lodish, 1993; Penton et al., 1994; Attisano et al., 1995; Liu et al., 1995; Yingling et al., 1995). These studies have proposed that binding of TGFβ to the constitutively active (autophosphorylated) TβR II recruits TβR I, which form a complex with the ligand-bound TβR II. Subsequent to formation of the

complex, TβR II phosphorylates TβR I on its serine/glycine rich (GS) domain, which seems to be essential for the downstream cytoplasmic responses induced by TGFβ.

Intracellular mediators of TGFβ signaling pathway

In recent years, efforts have been made by several investigators to recognize intracellular mediators of the TGFβ signaling pathway. Using a yeast two hybrid screening system to identify signaling molecules that directly interact with the cytoplasmic domain of TβR I, two intracellular TβR I-binding molecules, a peptidyl polyisomerase, FKBP12, and α subunit of farnesyltransferase (FT-α) have been recognized (Kowabata et al., 1995; Liu et al., 1995; Wang et al., 1994, 1996). Although the exact role of FKBP12 in TGFβ–induced signaling pathway is not yet known, it has been suggested that FKBP12 and TβR I may interact *in vivo* (Yingling et al., 1995). FKBP12 also binds to immunosupressive drugs, FK506 and rapamycin (Schrieber, 1991; Fruman, et al., 1994) to form a complex, which causes G1 arrest in cells by inhibiting cyclin dependent kinase activity required for G1 to S phase transition (Yingling, 1995; Polyak 1996). FT-α is the regulatory subunit of Ras farnesyltransferase which seems to play an important role in activation/modulation of Ras molecule, other members of Ras superfamily and γ subunit of G proteins, all of which are implicated in cell regulation (Hancock et al., 1989; Wang et al., 1996). Wang and colleagues (1996) indicated that FT-α binds ligand-free TβR I, and is subsequently phosphorylated and released in the cytoplasm. Its ensuing involvement in TGFβ–induced signaling pathway, however remains unclear.

Also, a highly conserved family of proteins, mothers against dpp (MAD) proteins, initially identified in *Drosophila* (Sekelsky et al., 1995; Newfeld et al., 1996; Wiersdorff et al., 1996) and subsequently in *C.elegans, Xenopus*, and mammals (Massagué, 1996; Hill, 1997) has been implicated as an intracellular mediator of the TGFβ signaling pathway. The

null mutants of MAD gene possess identical phenotype as mutants of dpp, a TGFβ related protein in *Drosphila* (Hoodless et al., 1996; Wiersdorff et al., 1996). It has been suggested that, upon trans-phosphorylation of TβR I with TβR II, MAD protein transiently interacts with TβR I cytoplasmic domain (Zhang et al., 1996; Macias-Silva et al., 1996). The interaction between activated TβR I and MAD protein results in MAD protein phosphorylation, and subsequent accumulation in the nucleus (Baker and Harland, 1996; Hoodless et al., 1996; Liu et al., 1996; Macias-Silva et al., 1996). The exact role of MAD proteins in the nucleus is not yet well defined. Liu et al. (1996), however, showed that when fused to the DNA binding protein, GAL-4 protein, the C-terminus of MAD acts as a transcriptional activator of GAL-4 reporter gene. Such observations may be suggestive of a possible involvement of MAD proteins in regulation of gene expression in response to TGFβ-induced signal transduction (Arora et al., 1995; Liu et al., 1996; Niehrs, 1996).

Antiproliferative actions of TGFβ

TGFβ seems to exert growth inhibitory effects in normal and transformed cell lines of epithelium, endothelium, fibroblast, neural, lymphoid, and haemopoetic cell types (Massagué, 1992). TGFβ has been suggested to exert its antiproliferative effects through various mechanisms during mid to late G1 phase of cell cycle (Massagué, 1990; Roberts and Sporn, 1990; Kingsley, 1994; Polyak 1996). One of the proposed mechanisms by which TGFβ inhibits cell cycle progression is through preventing the phosphorylation of retinoblastoma tumor suppressor protein (Rb) (Laiho et al., 1990; Polyak 1996). Rb is a key player in the cell cycle machinery and interacts with the cell cycle regulators, cyclins, Cdks, Cdk inhibitors, and cyclin-activating kinase (Brand and Schneider, 1995).

Another mechanism by which TGFβ may restrain cell proliferation is through modulation of the mitogenic signaling pathway involving Ras, Raf-1, MEK, and MAPK

(Kolodziejczyk and Hall, 1996; Polyak, 1996). Recent studies have shown that the effects of TGFβ on the Ras-MAPK pathway is highly dependent on cell type and environment. For instance, TGFβ treatment of variety of cell types such as intestinal epithelia (Mulder and Morris, 1992) , HD3 colon carcinoma (Yan et al., 1994), and CCL64 mink lung epithelia (Hartsough and Mulder, 1995) rapidly induces the activation of Ras protein. In addition, Howe and associates (1993) showed that microinjection of the oncogenic Ras protein (Ha-Ras) to TGFβ treated mink lung epithelial cells overcomes the TGFβ growth inhibitory effect, and allows progression of the cell cycle. On the other hand, microinjection of anti-Ras antibody to mink lung epithelial cells following release from TGFβ treatment causes the cells to remain in a growth arrested state (Howe et al., 1993). These observations suggest a possible involvement of Ras protein in the TGFβ-induced signaling pathway.

Furthermore, the ability of TGFβ to modulate the activity of MAPK is cell type specific effects. In proliferating cultures of intestinal epithelial cells (Hartsough and Mulder, 1995), HD3 colon carcinoma cells (Yan et al., 1994), and mesangial cells (Huwiler and Pfeilschifter, 1994), TGFβ treatment seems to activate various isoforms of MAPK. Conversely, in other cell types such as CCL64 mink lung epithelial cells (Hartsough and Mulder, 1995) and smooth muscle cells (Berrou et al., 1996), TGFβ treatment inhibits MAPK activation. Alternatively, TGFβ treatment has been reported to up-regulate serine-threonine phosphotases in some cell types (Gruppuso et al., 1991; Fontenay et al., 1992). In their study, Berrou et al. (1996) have hypothesized that TGFβ may display its anti-proliferative effects on FGF-induced smooth muscle cells through activating serine/threonine phosphatases that interfere with Ras-MAPK mitogenic pathway.

Another hypothesis, indicating direct involvement of TGFβ in Ras-MAPK through a TGFβ-activated kinase (TAK-1), has also been proposed (Yamaguchi et al., 1995). TAK-1 is a member of the MAPK kinase kinase (MAPKKK) family of protein kinases, which was

isolated from a c-DNA library of murine cells (Yamaguchi et al., 1995). The kinase domain of TAK-1 shows 30% homology with c-Raf and MEKK. Addition of TGFβ to MC3T3 osteoblasts seems to stimulate the kinase activity of TAK-1 within 5-10 minutes in a dose dependent manner (Yamaguchi et al., 1995). In addition, two TAK-1 binding proteins (TAB-1 and -2) have recently been isolated from the cDNA library of human brain cells, which seem to enhance TGFβ-regulated activity of TAK-1, implicating their potential involvement in the TGFβ signaling pathway (Shibuya et al., 1996).

Immediate Early Genes

Biological behavior of cells of multicellular organisms is regulated by both the proximate and distant environmental factors through ligand-receptor mediated transcriptional changes (figure 2). The ligand-receptor complex sets in motion a series of cross-talking, intracytoplasmic signaling cascades, which transmit signals to the nucleus, and activate transcriptional machinery to enforce gene expression. In a developing system, spatio-temporally specified, ligand-induced gene expression determines the biological behavior of cells, which, in turn, modulates the morphogenesis of a structure/organ. The ligand controlled behavior of cells during development may result from a combinatorial activation of a set of genes, whose temporal kinetics of quantitative induction, cell/tissue specific expression, and post-translational modifications of gene products plays an important role in regulating variations in the biological response (Herschman, 1991).

Following demonstrations by Riddle and associates (1979) that serum-induced mitosis was accompanied by a rapid increase in synthesis of nuclear and cytoplasmic proteins, and by Stiles and Colleagues (1979) that treatment of BALBc/3T3 quiescent fibroblasts by EGF and PDGF would rapidly induce mitosis in them, Herschman and Scher (1983) showed that both EGF and PDGF induce the accumulation of translatable RNA.

Subsequently, it was shown that a brief exposure to serum, EGF, FGF, or PDGF induces a rapid and transient transcription of *c-fos*, *c-jun*, and *c-myc* proto-oncogenes (Kelly et al., 1983; Bravo, et al., 1985; Ryseck et al., 1988; Quantin and Breathnach, 1988). These genes were named "immediate early genes" because following mitogenic stimulation of cells their expression was rapid (within minutes), often transient, and did not require *de novo* protein synthesis (Henrikson and Lüscher, 1996). Immediate early genes generally participate in normal cellular regulation involving signal transduction cascades, to convert extra-cellular messages through target genes into a program of gene expression (figure 2). The protein products of immediate early genes are involved in many cellular activities such as cellular growth, proliferation, differentiation and oncogenic transformation (Morgan and Curran, 1989).

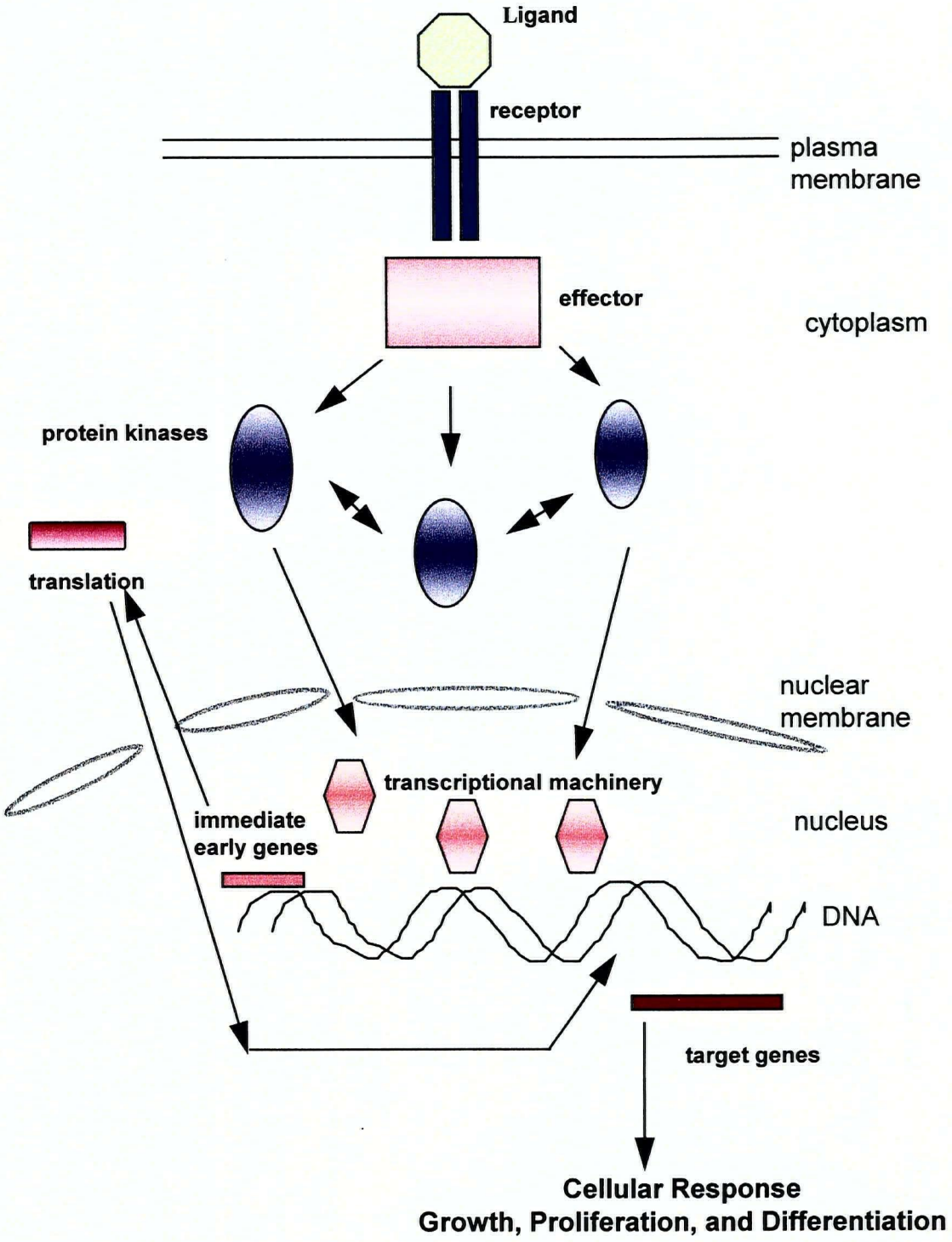
So far, over 170 immediate early genes have been identified in serum- or growth factor-treated cells, or from regenerating tissues (Mohn et al., 1991). A highly varied pattern of their expression in different cell types indicates that tissue specificity of their biological response may be related to a particular set of genes expressed in a given tissue or in response to an inducing agent rather than expression of a few cell type specific genes (Mohn et al., 1991).

Depending upon the cellular milieu, immediate early genes encode proteins that can act as activators of transcription factors or proteins involved in signal transduction cascades to regulate cell behavior (Hunter and Karin, 1992). To activate transcription of an immediate early gene, a transcription factor should localize into the nucleus, bind to DNA, and interact with the basal transcription factor apparatus (Hill and Triesman, 1995). One of the most common ways of transcription regulation is through phosphorylation by members of signaling pathways (Hunter and Karin, 1992). Phosphorylation of a transcription factor or its associated protein can induce conformational changes in the

protein to promote: its nuclear localization, its association with a coactivator protein, its dimerization, its transactivation, or its DNA binding properties (Hill and Triesman, 1995). Such phosphorylation-led changes eventually regulate the biological behavior of cells such as growth, proliferation, differentiation, and/or apoptosis (Pawson and Hunter, 1994).

In biological systems, the changes in immediate early gene expression appear to be associated with potential changes in cellular capabilities: new transcription factors are induced that could affect the expression of secondary (target) genes, thus linking acute stimuli with long term adaptive changes in cellular gene expression. The functional role of inducible genes in specific physiological systems however is not well defined. The change from a quiescence to proliferating state of cell is characterized by the induction of several waves of genes which are believed to be necessary for the onset and progression of cell cycle (Beserga, 1985). Many immediate early genes encode transcription factors that bind to specific DNA sequence elements present in the regulatory regions of their target genes, thus regulating a subsequent wave of gene expression. Three of these early response genes that are involved in cell cycle progression are the proto-oncogenes *c-fos, c-jun*, and *c-myc*. Because of their rapid and widespread transcription, these proto-oncogenes provide an excellent model for studying the mechanisms by which extracellular stimuli regulate DNA synthesis during the cell cycle. In the present study, the effects of growth factors on expression of these immediate early genes was analyzed to investigate their involvement in proliferation of embryonic palate mesenchymal cells.

Figure 2. Ligand-induced cellular response through activation of immediate early genes.

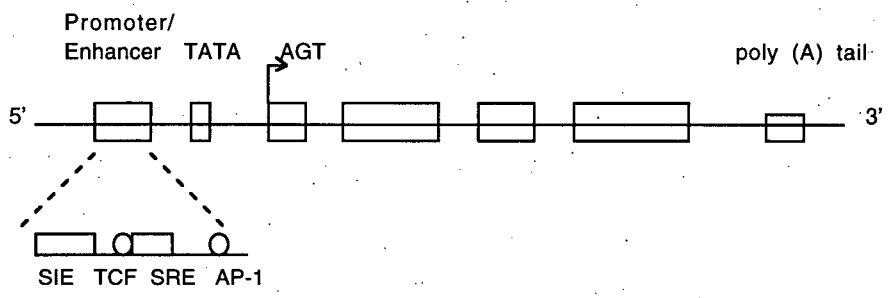


c-fos

The immediate early gene *c-fos* is one of the best characterized proto-oncogenes, and its protein product, c-Fos, belongs to a family of highly related nuclear phosphoproteins which also includes v-Fos, FosB, and Fos-related antigens, Fra-1 and Fra-2 (Verma and Graham, 1987; Curran, 1988; Distel and Spiegelman, 1990; Hesketh, 1994; Misra, 1994; Piechaczyk and Blanchard, 1994). *fos* was initially isolated, as the gene responsible for induction of bone tumors (*v-fos*), from two murine osteosarcoma retroviruses, Finkel-Biskins-Jinkins (FBJ) and Finkel-Biskins-Reilly (FBR), by Curran and Teich in 1982 (the term *fos* is derived from *F*BJ/FBR *os*teosarcoma) . Subsequently, cellular *fos* (*c-fos*) was cloned from a mouse liver and a human lymphoblast cell line (Curran et al., 1983) and its complete nucleotide sequence was determined (Van Beveren et al., 1983, 1984; Van Straaten et al., 1983). In addition to mice and humans, *c-fos* has been identified in other vertebrates such as chicken and *Xenopus*, and its structure seems to be highly conserved among various species (79-94%) (References cited above).

The *c-fos* gene structure consists of a 5' promoter region, a coding region containing four exons, and a 3' non-coding region containing poly(A) tail (figure 3a). The mRNA transcript of *c-fos* has a size of 2.2 kb as detected by Northern blotting, and encodes the 380 amino acid c-Fos molecule. The primary translation product of *c-fos* is 55 kDa (Curran et al., 1982). However, on polyacrylamide gel electrophoresis, the apparent molecular weight of c-Fos is between 55-62 kDa, perhaps due to high proline content of the protein and post-translational modifications such as phosphorylations and phosphoesterifications on its serine and threonine residues (Curran et al., 1984; Verma et al., 1984; Muller et al., 1987).

a) *c-fos* gene



b) c-Fos protein

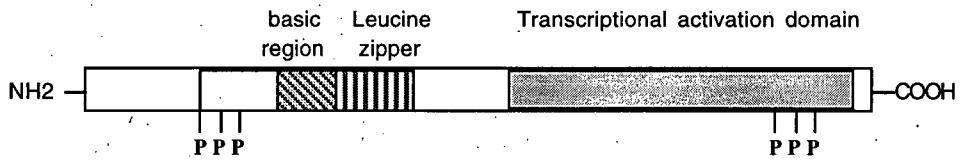


Figure 3. Schematic diagram of *c-fos* gene (a) and c-Fos protein (b) structure (source: Ransone and Verma, 1990).

c-Fos is a short lived transcription factor (half life 30 minutes-2 hours; Curran et al., 1984; Müller et al., 1984; Curran and Morgan, 1986), which has been implicated in a variety of cellular activities including cell growth, proliferation, differentiation, death and oncogenic transformation (Verma, 1986; Gonzalez-Martin et al., 1992). Structurally, c-Fos consists of a leucine zipper region, a highly basic region, and a transcription activation domain (figure 3b) (Curran, 1988; Distel and Spiegelman, 1990). The phosphorylation of c-Fos seems to be altered by a variety of extracellular stimuli. *In vitro* studies have shown that both the C-terminal and the N-terminal domains of c-Fos can be phosphorylated by several protein kinases such as p34-(cdc2), PKA, PKC, MAP kinase, DNA dependent protein kinase (DNAPK), GSK, and RSK (Abate et al., 1991; Taylor et al., 1993). Extensive post-transcriptional modifications of c-Fos led to suggestion that c-Fos activity may be a distal intermediate in the process of signal transduction (Distel and

Spiegelman, 1990). c-Fos could translate diverse short-term events from cell membrane into both short-term and long-term changes in gene expression (Morgan and Curran, 1986). The exact effect of c-Fos phosphorylations in regulation of its transcriptional activity is, however, not well understood.

To act as a transcription factor, Fos molecules require dimerization with the Jun family of transcription factors. Due to their protein structure, Fos members are not capable of forming homodimers, and therefore, on their own, they cannot bind to DNA and activate transcription (Verma and Graham, 1987; Curran et al., 1993; Piechaczyk and Blanchard, 1994).

Regulation of *c-fos* transcription

Several extracellular stimuli such as serum, growth factors (EGF, PDGF, NGF, FGF, etc.), cytokines, cAMP, Ca^{2+}, phorbol esters, UV light, etc., may result in rapid and transient induction of *c-fos* in a variety of cell types including fibroblasts, lymphocytes, nerve cells, and established cell lines (Curran, 1988; Distel and Spiegelman, 1990; Ransone and Verma, 1990; Angel and Karin, 1991). *c-fos* transcription usually begins within minutes after stimulation of the responding cells; its mRNA levels reach maximum at 30-60 minutes and decline to basal levels by 90-120 minutes (Cochran et al., 1988; Curran, 1988; Misra, 1994). It is generally agreed in the literature that *c-fos* transcriptional activation involves several complex regulatory mechanisms.

One of the mechanisms responsible for *c-fos* transcriptional regulation involves interaction of several transcription factors with the *c-fos* promoter region (Curran, 1988). The *c-fos* promoter region contains a number of regulatory sequences (Hill and Treisman, 1995; Janknecht, 1995) (figure 4). The serum response element (SRE) is a protein binding site required for the induction of *c-fos* expression by serum and mitogens

(Gilman et al, 1986; Treisman, 1992). SRE appears to be constitutively occupied by a ternary complex of transcription factors that contains serum response factor (SRF) homodimer, and a ternary complex factor (TCF; which includes Elk-1, SAP-1, or SAP-2) (Norman et al., 1988; Shaw et al., 1989). Following stimulation of quiescent cells by serum or growth factors, both SRF and TCF are phosphorylated (Prywes et al., 1988; Janknecht et al., 1993; Marais et al., 1993). The exact mechanism responsible for activation of SRF is not well known; however, involvement of PKC dependent pathways has been implicated (Graham and Gilman, 1991). Furthermore, phosphorylation of TCF molecules by MAP kinase seem to play an important role in stimulation of *c-fos* expression (Shaw et al., 1989; Hipskind et al., 1991; Hill et al., 1993; Davis, 1994). Another regulatory DNA sequence in the *c-fos* promoter is the calcium and cAMP response element (CRE), which mediates rapid *c-fos* induction in response to elevated cAMP and calcium (Gilman, 1986; Sassone-Corsi et al., 1988; Sheng et al., 1988; Fisch et al., 1989). Expression of *c-fos* in response to calcium and cAMP has been proposed to occur through phosphorylation of CRE binding protein (CREB) by PKA (Sheng et al., 1991). The *sis*-inducible element (SIE) is also a transcription factor binding site in the *c-fos* promoter. SIE seems to interact with the STAT family of transcription factors and contribute to *c-fos* promoter activation by cytokines and growth factors that induce STAT DNA-binding activity (Fu and Zhang, 1993; Sadowski et al., 1993; Zhong et al., 1994; Leaman, et al., 1996). It has been suggested that an AP-1 binding region in the promoter region of *c-fos* may be responsible for *c-fos* negative auto-regulation (Sasson-Corsi et al., 1988; Fisch et al., 1989).

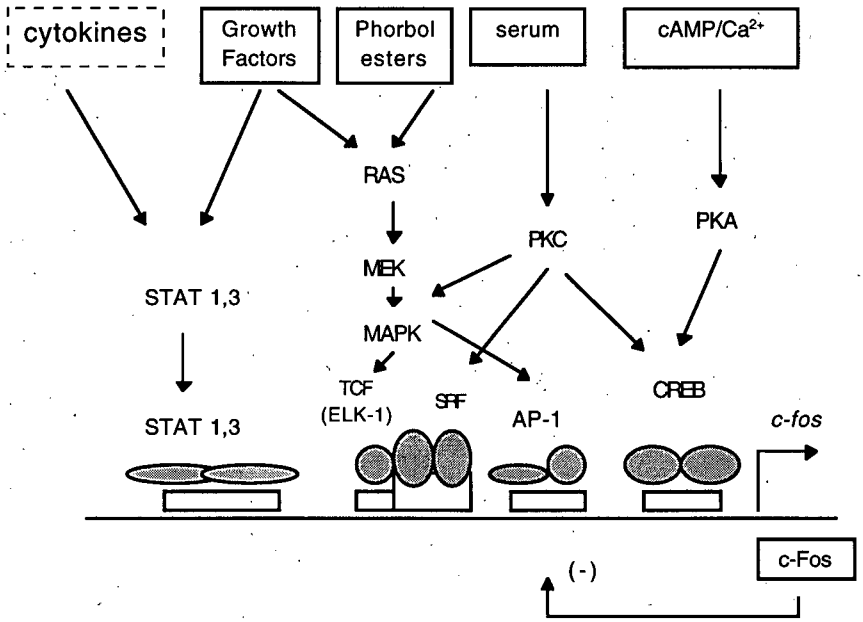


Figure 4. Schematic diagram of *c-fos* transcriptional regulation on its promoter region (modified from: Hill and Triesman, 1995).

In addition to promoter-directed regulatory mechanisms, other mechanisms appear to be involved in regulation of *c-fos* transcription. The rapid turn over of the *c-fos* mRNA seems to depend on the presence of a AT-rich untranslated region at the 3' of *c-fos* as well as a region in the coding domain of *c-fos* (Meijlink et al., 1985; Rahmsdorf et al., 1987; Lee et al., 1988; Raymond et al., 1989). In the presence of protein synthesis inhibitors, the half life of *c-fos* mRNA is increased, suggesting an involvement of a rapidly induced RNase in the degradation of these molecules. Also, c-Fos may be involved in down-regulation of its own transcription. Sasson-Corsi and associates (1988a), and Schonthal and colleagues (1988) indicated that over expression of *c-fos* may result in rapid reduction of both the basal level and serum induced levels of c-fos expression. Similarly, inhibition of *c-fos* protein synthesis using antisense RNA seem to lead to an increase in the *c-fos* transcription. Further analysis of *c-fos* deletion mutations revealed that the C-terminal of c-Fos may be involved in its transcriptional repression (Gius et al., 1990).

c-jun

The proto-oncogene *c-jun* is another immediate early gene, which has been the subject of extensive investigations during the past two decades. The protein product of *c-jun*, c-Jun is a member of a family of related transcription factors that also includes v-Jun, JunB, and JunD. Although the Jun transcription factor family members share significant sequence homology, they are expressed in variable amounts in different cell types and tissues, and show different transcriptional and biological activities (Chiu et al., 1989; Schutte et al., 1989; Castellezzi et al., 1991; Deng and Karin, 1993; Pfarr et al., 1994). *jun* was initially identified and isolated as the transforming gene of avian sarcoma virus 17 (ASV 17) in chicken cells by Maki and associates and Vogt and colleagues in 1987 (the term *jun* is the condensed form of "*jun*ana", the Japanese word for 17). Subsequently, cellular *jun* has been identified in several vertebrates (humans, mice, rats, and chickens) where it shows to have high sequence homology (71-99%) across species (Ryder et al., 1988; Schutte et al., 1989; Nomura et al., 1990; Hartl et al., 1991).

The vertebrate *c-jun* gene consists of a 5' promoter region, a single exon without any introns, and an extensive 3' non-translated region (figure 5a). It encodes for 330 amino acid (39kDa) c-Jun protein. Using Northern blotting technique, the mRNA transcript of c-*jun* has been detected in two sizes 2.7 and 3.3 kb. The two transcripts seem to differ in the size of untranslated poly(A) (AU-rich sequence) tail at the 5' end of the mRNA (Ransone and Verma, 1990; Vogt and Bos, 1990; Hesketh, 1994).

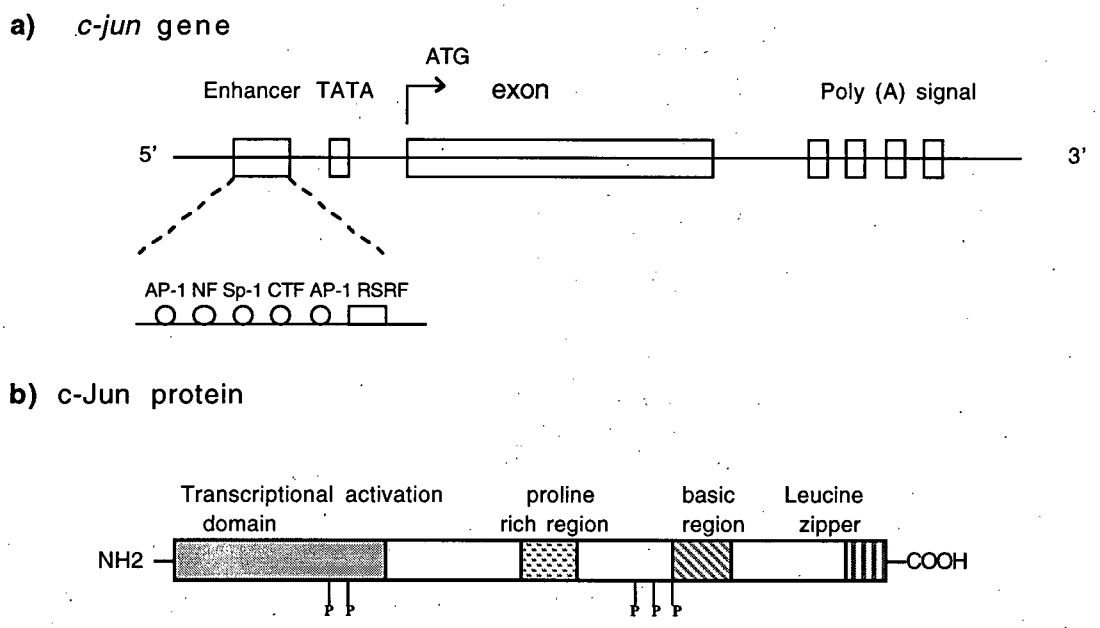


Figure 5. Schematic structure of *c-jun* gene (a) and protein (b) (source: Ransone and Verma, 1990).

The product of proto-oncogene *c-jun* has been implicated in regulation of a variety of cellular activities including cell proliferation, differentiation, death, and oncogenic transformation (Vogt and Bos, 1990; Angel and Karin, 1991; Devary et al., 1991; van Dam et al., 1995; Bossy-Wetzel et al., 1997). Structurally, c-Jun protein consists of a leucine zipper domain and a highly basic domain in its C-terminal region and a highly acidic transcriptional activation domain in its N-terminal region (figure 5b). *In vivo* and *in vitro* studies have revealed that both the C-terminal and the N-terminal domains of c-Jun can be phosphorylated by several protein kinases including Jun-N-terminal kinase (JNK), stress activated protein kinase (SAPK), GSK, and CK 2 (Devary et al., 1992; Karin and Smeal, 1992; Hibi et al., 1993). In resting (G0) epithelial and fibroblast cells, c-Jun is phosphorylated by GSK on the C-terminal near its DNA binding domain, which exerts

inhibitory effects on c-Jun activity. In growth factor or mitogen stimulated cells, activation of c-Jun occurs through dephosphorylation of the C-terminal (perhaps through a PKC dependent mechanism) and phosphorylation of the N-terminal, by protein kinases such as JNK, SAPK, and CK2 (Boyle et al., 1991; Hunter and Karin, 1992, Pulverer et al., 1993).

To act as an active transcription factor, the c-Jun molecule should form a dimer complex with other transcription factors. All the members of Jun family, including c-Jun associate with Fos proteins to form Fos-Jun heterodimers, also known as activator protein-1 (AP-1). In turn, the AP-1 family of transcription factors activate a wide assortment of genes in different types of cells in response to the environmental stimuli that activate signal transduction pathways (Hunter and Karin, 1992; Hill and Triesman, 1995; Karin, 1995). To activate a gene, the AP-1 molecules bind to 5'TGAG/CTCA3' consensus sequences on DNA, recognized as the TPA (12-O-tetradecanoylphorbol 13-acetate) response element (TRE) of several cellular and viral genes (Angel and Karin, 1991). The affinity of Jun protein for DNA binding is significantly increased by the presence of Fos. In addition, unlike c-Fos, c-Jun homodimer acts as an active transcription factor; however, its activity is much less than Jun/Fos heterodimer (Angel and Karin, 1991). In addition to c-Fos, c-Jun also associates with other transcription factors such as ATF-2 and CREB to form active transcription factor heterodimers (Hai and Curran, 1991). The ability of c-Jun to interact with different transcription factors may result in its binding to several distinct DNA binding sites and activation of diverse groups of genes.

Regulation of *c-jun* transcription

The *c-jun* gene is expressed in response to a variety of extracellular stimuli including growth factors, UV light, phorbol esters, oxidative stress, etc. (Sherman et al.,

1990; Devary et al., 1991; Rozek and Pfeifer, 1995). In most cell types, the *c-jun* mRNA levels increase within 30-60 minutes following stimulation, and decline to basal levels by 2-4 hours (Lamph et al., 1988; Ryder and Nathans 1988). Elevation of *c-jun* mRNA levels appears to be due to an increase in gene transcription in response to extracellular stimuli (Ryder and Nathans, 1988; Sherman et al., 1990; Devary et al., 1991; Bergelson et al., 1994). Several transcription factor binding regions have been identified in *c-jun* promoter region, which seem to be responsible for regulating its expression (figure 6). These regions include a serum response factor-related binding domain (RSRF), two AP-1-like binding domains (jun1 and jun2), a CAT domain, a SP-1 domain, and a nuclear factor-Jun binding domain (NF-Jun).

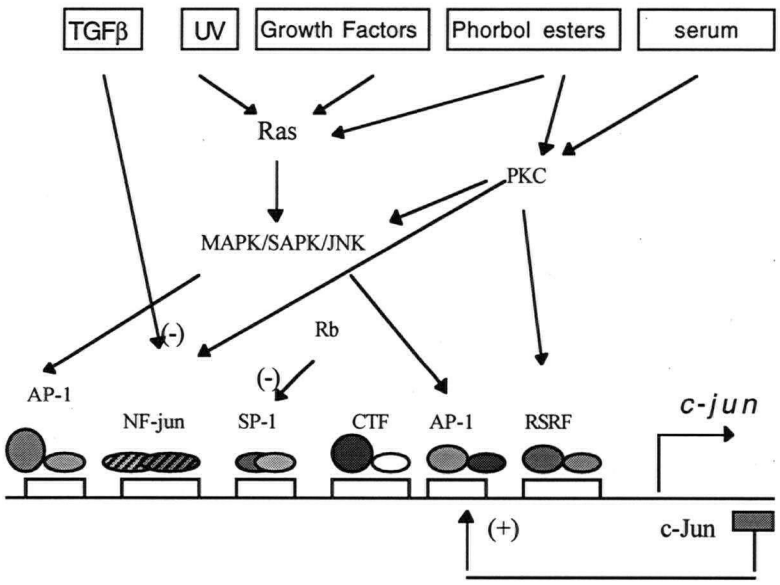


Figure 6. Schematic diagram of *c-jun* transcriptional regulation.

In both unstimulated and stimulated fibroblasts, the *c-jun* promoter region is occupied by transcription factors; therefore, mechanisms such as post-transciptional modifications of the transcription factors (eg. phosphorylation by various signaling pathways) or replacement of less active transcription factors with more active ones may be responsible for induction of c-jun expression (Rozek and Pfiefer, 1993; Herr et al., 1994; van Dam et al., 1995). Both the AP-1 binding sites in *c-jun* promoter seem to be involved in positive regulation of c-jun expression by serum, EGF, UV light and phorbol ester (Angel et al., 1988; Han et al., 1992; van Dam et al., 1993; Herr et al., 1994). The transcription factors that interact with *c-jun* AP-1 sites include c-Jun/AP-1 and c-Jun/ATF-2 complexes (figure 6). *In vivo* and *in vitro* studies have shown that several different mitogen induced protein kinase pathways (including MAP kinase signaling cascade) are able to phosphorylate c-Jun at both at the transcriptional activation domain and at the DNA binding domain (Pulverer et al., 1991; Baker et al., 1992; Hibi et al., 1993; Kamada et al., 1994) to increase its transcriptional activity (Smeal et al., 1992; Hibi et al., 1993). ATF-2 also appears to be a target of stress activated protein kinase (SAPK), a member of the MAP kinase family (figure 6), in response to UV light stimulation (van Dam et al., 1995). RSRF seems to be responsible for *c-jun* induction by serum, phorbol esters, and EGF in fibroblast cells (Han et al., 1992; Rozek and Pfeifer, 1995). The mechanism by which extracellular stimuli induce RSRF transcriptional activity, however, is unknown. Nuclear factor-jun (NF-jun) binding region associates with NF-jun transcription factor, which has several features similar to NFKB and its expression is restricted to proliferating cells (Brach et al., 1992). NF-jun seems to be involved in activation of *c-jun* transcription in response to tumor necrosis factor-α (TNF-α) and phorbol esters. The PKC (figure 6) pathway has been proposed to regulate NF-jun transcriptional activity (Brach et al., 1992). In addition, RB protein has been reported to activate *c-jun* expression in

fibroblasts (Chen et al., 1994). RB protein seems to exert its effect on *c-jun* expression through binding to the Sp-1 binding site in the *c-jun* promoter as well as through binding to the Sp-1 inhibitor and result in release of active Sp-1 transcription factor (Chen et al., 1994).

In addition to the positive regulatory mechanisms, *c-jun* expression is subject to several negative regulatory mechanisms. *c-jun* negative regulation seems to be important in normal cell function, since *c-jun* over-expression may result in oncogenic transformation. Similar to other immediate early gene, *c-jun* mRNA transcripts are very unstable. The *c-jun* mRNA has a very long untranslated poly (A) tails (approximately 1kb), which may be the site of specific RNAse enzymes (Hattori et al., 1988). Additional negative mechanisms may also operate through *c-jun* promoter region. For example, transfection experiments have shown that homodimers of JunB, a c-Jun-related proto-oncogene, bind to AP-1 binding sites in *c-jun* promoter and act as its negative regulator (Angel and Karin, 1991). Furthermore, transcriptional activity of *c-jun* may be repressed by CREB, which forms heterodimers with c-Jun and bind to the AP-1 binding region (Angel et al., 1988; Benbrook and Jones, 1990). The activity of CREB seems to be regulated by its phosphorylation through PKA in response to cAMP- inducing factors (Benbrook and Jones, 1990; Macgregor et al., 1990). In some cell types TGFβ seems to exert negative effects in activation of *c-jun* expression (figure 6). Sott and associates (1994) suggested that TGFβ exert its negative effects by inhibition of the nuclear activity of NF-jun transcription factor. Taken together, these findings show that transcriptional regulation of *c-jun* is a complex process, and involves interaction of a large number of regulatory proteins.

c-myc

One of the most extensively studied immediate early genes is the *c-myc* proto-oncogene, whose protein product, c-Myc, belongs to a large family of highly related phosphoproteins that also includes v-Myc, N-Myc, B-Myc, S-Myc and L-Myc (Marcu et al., 1991; Spencer and Groudine, 1991; Henriksson and Lüscher, 1996; Lemaitre et al., 1996). Upon translation in the cytoplasm, these proteins have been shown to translocate to the nucleus, bind to specific DNA sites, and act as transcriptional activators. In 1979, Shieness and Bishop identified the first *myc* gene, viral *myc*, (*v-myc*), as the transforming sequence in the avian leukemia retrovirus MC29, whose expression caused *my*elo*c*ytomas, carcinomas, sarcomas, and lymphomas in birds fibroblasts and macrophages (Cole, 1986). In 1982, Vennstrom and associates isolated the cellular homologue of *v-myc, c-myc,* from chicken fibroblasts. It is now recognized that *c-myc* is evolutionarily conserved; its homologue genes have been cloned and characterized in insects, zebra fish, frogs, sea stars, as well as in mammals (Marcu et al., 1992; Henriksson and Lüscher, 1996; Lemaitre et al., 1996).

The gene structure of *c-myc* consists of a promoter region, three exons, and a poly(A) tail (figure 7a). The *c-myc* gene product is translated from exons two and three. The exon one is non-coding although it is evolutionarily conserved (Farhlander and Marcu, 1986; Marcu et al, 1992). Using immunoprecipitation techniques, Hann and associates (1983) and Personn and coworkers (1984) detected translation of at least two nuclear proteins p64 and p67 from human *c-myc*. These two gene products seem to exhibit very similar phosphorylation, protein interaction and DNA binding properties in both *in vivo* and *in vitro* systems (Personn et al., 1984; Ramsay et al., 1984; Watt et al., 1985). The transcripts for these two gene products have a size of 2.4 and 2.2 kb and appear to be

encoded under the direction of two different promoters, P1 (10-25%) and P2 (75-90%) (Henriksson and Luscher, 1996; Lemaitre et al., 1996).

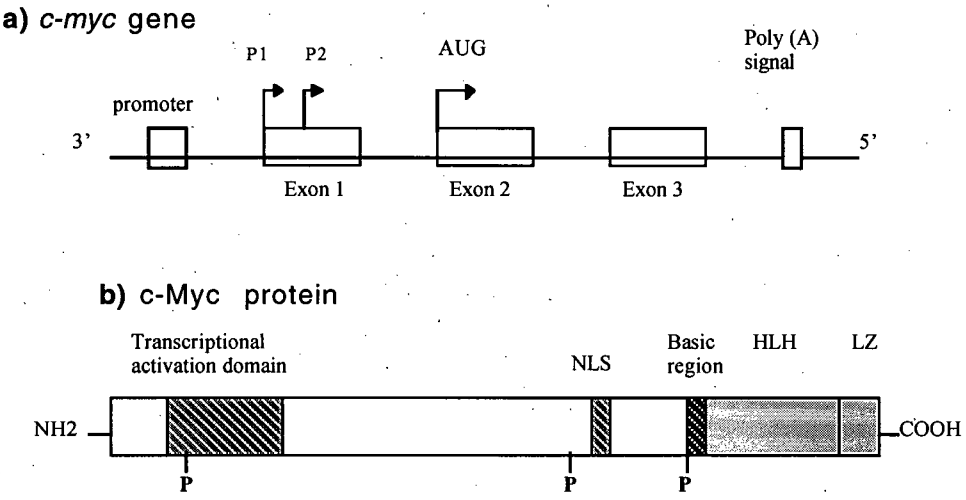


Figure 7. Schematic diagram of *c-myc* gene (a) and c-Myc protein (b) structures (source: Marcu, et al., 1992; Lemaitre et al., 1996).

Structurally, c-Myc consists of a transcriptional activation domain in its N-terminal (Kato et al., 1990), a basic helix-loop-helix leucine zipper (bHLHLZ) domain (Landschulz et al., 1988; Murre et al., 1989; Lüscher and Eisenman, 1990), as well as two nuclear localization signals (NLS) in its C-terminal (Dang and Lee, 1988) (figure 7b). Numerous *in vivo* and *in vitro* studies have shown that c-Myc is phosphorylated on serine and threonine residues in the transcriptional activation domain, by glycogen synthase kinase-3 (GSK-3), MAP kinase, p34 cdc2 kinase (CDK1), and a p107/cyclinA/CDK complex, and in the C-terminal domain by Casein kinase-2 (CK-2) (Lutterbach and Hann, 1994; Henriksson and Luscher, 1996; Lemaitre, et al., 1996). Phosphorylation of c-Myc on serine and threonine residues is regulated by mitogens, and alters its ability to induce gene transcription. Also, it has recently been shown that the phosphorylation sites of c-Myc

are different in immortalized and transformed cell lines compared with primary cells (Lutterbach and Hann, 1997). c-Myc is a short-lived protein with a half life of 20-30 minutes (Lüscher and Eisenman, 1990). From a functional viewpoint, c-Myc is implicated as a positive regulator of cell proliferation, cell cycle progression, neoplastic cell transformation, and apoptosis, and an inhibitor of cell differentiation (Marcu et al., 1992; Pakham and Cleveland, 1995; Herinksson and Lüscher, 1996).

To function as a transcription factor, dimerization of c-Myc with another protein is essential. c-Myc homodimers are unstable and seem to be physiologically inactive. The c-Myc partner, Max, was identified by screening a human cDNA expression library with a radiolabelled fusion protein containing the c-Myc C-terminus (Blackwood and Eisenman, 1991). Max is a bHLHLZ transcription factor that lacks a transcriptional activation domain and forms stable heterodimers with c-Myc, N-myc, and L-Myc as well as homodimers with itself (Blackwood and Eisenman, 1991; Wenzal et al., 1991; Blackwood et al., 1992; Mukherjee et al., 1992). Also, unlike *c-myc*, Max protein is abundant in various cell types, has a long half life, and its expression is not regulated by growth factors or mitogens (Amati and Land, 1994).

Myc and Max interact with each other through their HLH and LZ domains, and with DNA through their highly basic regions (Crouch et al., 1993; Davis and Halazonetis, 1993). Myc/Max heterodimers bind to a DNA concensus hexamer sequence, CACGTG, also known as E-box (Blackwell et al., 1990; Prendergast et al., 1991, Fisher et al., 1991; Kerkhoff et al., 1991). Since recognition of the E-box, several studies have focused on characterizing the genes that possess this E-box in their promoter and are subsequently induced by the c-Myc/Max dimer. The c-Myc/Max-induced target genes include: *α-prothymosin* (a nuclear protein with unknown function) (Eilers et al., 1993); ornithine decarboxylase (*ODC*) (Bello Fernandez et al., 1993; Tobias et al., 1995); tumor suppressor gene *p53*

(Riesman et al., 1993); a developmentally regulated gene *ECA39*, which may be involved in cell cycle regulation (Bevenisty et al., 1992; Schuldiner et al., 1996); *cad* which encodes one of the mediators of pyrimidine synthesis, (Miltenberger et al., 1995); cdc25A gene whose product is a CDK-activating phosphatase (Galaktionov et al., 1996), and *elf-2α*, encoding eukaryotic translation initiation factor (Rosewald et al., 1993). Even though the mechanism by which these target genes may mediate the effects of *c-myc* is not well understood, a few of these genes such as cad, ODC, and ECA39 have been suggested to be involved in cell cycle progression and cell transformation (Moshier et al., 1993, Miltenberger, 1995; Schulinder et al., 1996).

In addition to Max, two other bHLHLZ proteins, Mad (Ayer et al., 1993) and Mxi (Zervos et al., 1993), which interact with Max, but show no homology to either Myc or Max have been identified. It appears that these proteins compete with Myc for binding to Max with approximately equal affinities (Ayer et al., 1993). Recently, it has been proposed that Myc-Max-Mad may form a transcription factor network for controlling cell cycle progression, differentiation, and apoptosis (figure 8). In this network, Myc/Max heterodimers seem to induce cell proliferation and apoptosis, whereas Max/Mad and Mxi/Mad may be involved in growth arrest and cell differentiation (Amati et al., 1994; Henriksson and Lüscher, 1996). Whether various growth stimulatory factors that are implicated in modulation of c-Myc activity affect the transcription factor pairing is not yet known.

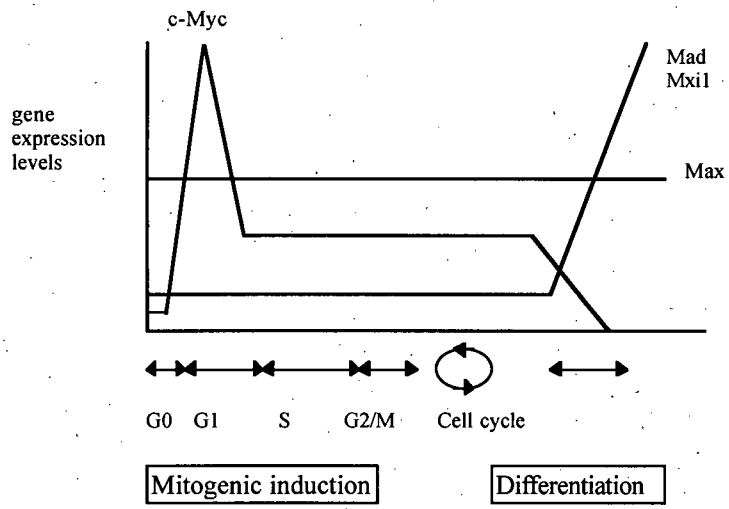


Figure 8. Myc-Max-Mad: Cell cycle and expression of Myc and associated proteins (source: Henriksson and Luscher, 1996; Lemaitre et al., 1996).

Regulation of *c-myc* transcription

The expression of the *c-myc* gene is highly regulated by extracellular factors. In quiesent cells, *c-myc* mRNA and protein are generally not readily detectable. Several growth promoting factors such as serum, PDGF, FGF, EGF, IL-3, CSF, etc. induce a rapid and transient *c-myc* expression in fibroblasts, keratinocytes, and lymphocytes (Marcu et al., 1992; Henrikson and Lüscher, 1996). In contrast, growth inhibitory, or differentiation promoting factors such as TGFβ, TNFα, and interferons appear to inhibit *c-myc* activation. For example, TGFβ downregulates mRNA and protein levels of *c-myc* in mouse BALB/MK keratinocytes (Polyak, 1996). Numerous studies have proposed the involvement of multiple growth factor-initiated signaling cascades in regulation of *c-myc* expression including PKC, c-AMP (PKA), JAK/STAT, CK2, and Src pathways (Luscher et al., 1989; Barone and Courtneidge, 1995; Lemaitre, et al., 1996; Watanabe et al., 1996). Furthermore, a number of regulatory sequences have been identified in the 5' flanking region (promoter region), exon 1, and possibly intron 1 of *c-myc*, which seem to interact

with transcription factors c-Myb, NF*K*B, Sp-1, NF-1, AP-2, E2F, AP-1, and octamer binding factor and may be involved in regulation of *c-myc* expression in response to the extracellular stimuli (Marcu et al., 1992; Dubik et al., 1996).

Another mechanism by which *c-myc* transcription is regulated involves its mRNA elongation. Abnormal transcript elongation has been noted in leukemia virus-transformed murine fibroblasts, differentiating mouse erythroleukemia (MEL) cells, and P19 cells (Nepveu, et al., 1987; St-Arnaud, et al., 1988). Also, it has been suggested that transcription elongation blockage may be a mechanism for down regulation of *c-myc* during differentiation (Campisi et al., 1984; Marcu al., 1992). Truncated transcripts do not seem to accumulate in the nucleus or cytoplasm of mammalian cells and are quickly destroyed in the nucleus (Spencer and Groudine, 1990). Several studies have investigated the regions necessary for transcription blockage within the *c-myc* gene. These regions seem to lie in the 3' end of the exon 1, P2 promoter, and 5' end of exon1/intron1 boundary (Miller et al., 1989; Wright and Bishop, 1989).

In human T lymphocytes, mouse spleen lymphocytes and T cells, and mouse fibroblasts, mitogens and growth factors such as PMA, Con A, and EGF have been suggested to exert their effect on *c-myc* transcription by relieving the block of elongation (Eick et al., 1987; Nepveu et al., 1987; Heckford, et al., 1988; Lindsten, et al., 1988; Curty et al., 1989). However, the exact mechanism of *myc* transcription blockage and its removal remains to be determined.

Post transcriptional modification of *c-myc* mRNA transcripts has been proposed to be yet another mechanism for regulating *c-myc* transcription. Alterations in *c-myc* mRNA stability was first identified in the malignant cells of murine plasmacytomas and Burkitt's lymphoma. In these cells, *c-myc* mRNA seems to be about 10 times more stable than in untransformed cells, with a half life of several hours instead of 10-20 minutes (Eick et al.,

1985; Piechanczyk et al., 1985; Rabbits et al., 1985). Also, post-transcriptional phenomena have been shown to contribute to both promotion and inhibition of *c-myc* transcription during cell proliferation and differentiation. For instance, post-transcriptional regulation seems to be responsible for increased levels of *c-myc* expression and mRNA stability in growth factor treated cells (Blanchard et al., 1985; Levine et al., 1986; Lacy et al., 1989). Furthermore, in regenerating kidney and liver *in vivo*, alterations in *c-myc* mRNA stability is responsible for enhanced *c-myc* induction (Asselin and Marcu, 1989; Sobczak et al., 1989; Morello et al., 1990). Several deletion/transfection studies have revealed that both 5' flanking/exon 1 sequences and an AU rich region at the 3' untranslated end of *c-myc* mRNA are important in determining the stability of *c-myc* transcript (Jones and Cole, 1987; Brewer, 1991).

The aforementioned mechanisms of *c-myc* transcriptional control indicate that regulation of c-myc proto-oncogene expression is a highly complex process. Differentiated cells tend to show reduced *c-myc* transcriptional initiation and premature transcription termination, whereas proliferating cells demonstrate an increased *c-myc* expression through a combination of enhanced transcriptional initiation and post transcriptional mRNA stabilization.

Furthermore, *c-myc* has been suggested to have an autoregulatory effect on its own gene expression. Using transgenic systems, Grigani et al (1990), and Penn et al (1990) have proposed a negative autoregulatory loop for *c-myc* in cells derived from primary cultures and established cell lines. In these cells, exogenous *c-myc* expression negatively regulates the endogenous c-myc expression in a dose dependent manner (Grigani et al., 1990). On the other hand, transformed cell lines might have lost their *c-myc* autoregulation ability (Grigani et al., 1990).

Immediate Early Genes in Cell Proliferation and Cell Cycle

The induction of *c-fos*, *c-jun*, *c-myc* transcription is one of the earliest nuclear response to a wide variety of extracellular stimuli, which are known to exert effects on cell proliferation, differentiation, transformation, and apoptosis. Depending upon the cell type, its differentiation state, and the specific environment, the effects of these proto-oncogenes on regulation of these biological processes appear to be varied.

Observation that: 1) the rapid and transient induction of *c-fos* and *c-jun,* during G0-G1 in cell occurs in response to various external stimuli that promote cell proliferation, and 2) induction of transformation of cell upon deregulation of these genes, led to the proposition that both these transcription factors may be required in regulation of cell cycle (Bravo and Muller, 1986; Lamph et al., 1988; Ryder and Nathans, 1988; Ryseck et al., 1988; Carter et al., 1991) as well as for the maintenance continuous cell proliferation (Smith and Prochownick, 1992). Also, purified antibody against *c-jun* has been shown to prevent DNA synthesis in fibroblasts (Kovary and Bravo, 1991).

The notion that the increased expression of *c-fos* and *c-jun* is necessary for the G0-G1 transition has been, however, challenged by the observation that cell proliferation may occur 1) in the absence of increased expression of these proto-oncogenes (Columbano and Shinozuka, 1996), or 2) in the presence of purified antibodies or antisense RNA against *c-fos* products (Kovary and Bravo, 1991). It has been, however, suggested that Fos related proteins may play compensatory roles in the absence of c-Fos (Kovary and Bravo, 1991).

A dimer combination between c-Fos and c-Jun forms activator protein 1 (AP-1) transcriptional complex, which is involved in transmitting growth promoting signals for cell proliferation and differentiation (Angel and Karin, 1991) as well as for apoptosis

(Smyene et al., 1993; Goldstone and Levine, 1994; Estus et al., 1994). AP-1 activates several genes in response to extracellular agents that stimulate various signal transduction pathways, leading to DNA synthesis (Hunter, 1987; Hunter and Karin, 1992; Hill and Triesman, 1995). It has been suggested that messages from different signaling pathways converge at AP-1 to eventually regulate its function (Bandyopadhyay and Faller, 1997).

Also, *c-myc* is rapidly induced within 1-2 hours of mitogenic stimulation of resting (G0) cells. Similar to other immediate early genes, mitogen induced expression of *c-myc* does not require protein synthesis (Cochran et al., 1983; Greenberg et al., 1985). Expression of *c-myc* alone seems to be necessary, and in some cases sufficient for G0-G1 transition in many cell types (Zoring and Evan, 1996). Unlike other immediate early genes, however, *c-myc* mRNA levels do not drop to the background levels after cell entry to G1, but they stay at a constant level throughout the cell cycle in proliferating cells (Henrikson and Luscher, 1996; Lemaitre et al., 1996). The continued expression of *c-myc* throughout the cell cycle may be suggestive of its role in other stages of the cell cycle in addition to G0-G1 transition. In fact, several studies have provided evidence that c-Myc may be required for S-to-G2-to-M transitions in many cell types (Waters et al., 1991; Shibuya et al., 1992; Seth et al., 1993; Born et al., 1994). Cells that express high levels of c-Myc protein generally have: reduced growth factor requirements (Armelin et al., 1984; Stern et al., 1986), high growth rates (Palmieri et al., 1983), and can overcome growth arrest (Armelin et al., 1984; Kohl and Raley, 1987).

Immediate Early Genes and Embryonic Development

Information on genes involved in regulation of cell growth, proliferation, and differentiation, during embryogenesis is of basic importance to understand the molecular

basis of development. All cells need continuous availability of different molecules to perform various biological activities. The transcription machinery, involving interaction between transcription factors and genes, is crucial in regulating timely synthesis and degradation of molecules by a cell for its biological activities of growth, proliferation, and differentiation (Davidson, 1986). Also, stimulation of a cell by diverse factors commits the cell to a complex (genetic) developmental program during which immediate early genes (proto-oncogenes) are transcriptionally activated within minutes in response to stimulation. During embryogenesis, immediate early genes are important not only because of their connections with extra-cellular factor-induced signal transduction pathways, but also because of their potential role in cellular determination and/or differentiation.

Fos, Jun, and Myc are all transcriptional regulators, and have been localized in various structures of vertebrate embryos or in cultured embryonic cells. c-Fos has been recognized during development of adipocytes and is expressed transiently in response to many extra-cellular stimuli (Lee et al., 1996). Also, Smeyne et al. (1993) and Yano et al. (1996a) have identified expression of *c-fos* in the epithelial cells during palate closure. c-Jun was localized during hepatogenesis (Hilberg et al., 1993), tooth development (Kitamura and Tarashita, 1997), and is implicated in long term maintenance embryonic fibroblasts (Vandel et al., 1996). Embryos lacking *c-jun* die during mid to late gestation or show retarted growth (Hilberg and Wanger, 1992; Hilberg et al., 1993; Johnson et al., 1993), indicating that *c-jun* is essential for normal embryonic development. No correlation between expression of *c-fos* or *c-jun* and biological behavior of cells was noted in these cells/tissues.

c-myc is perhaps the most widely studied immediate early gene during embryogenesis. It has been localized in developing eye, mandible, maxilla, and tooth

(Yamada et al., 1992), embryonic mesenchyme (Jaffredo et al., 1989; Stanton et al., 1992), chick limb (Ros et al., 1995), chondrocytes (Farquharson et al., 1992), myoblasts (Miner and Wold, 1991), and feather germ development (Desbiens et al., 1989). High level of N-*myc* expression during early development and *c-myc* expression during mid-gestation correlate with cell proliferation, whereas that of N- and L-*myc* with post-mitotic cells undergoing differentiation (Zimmerman et al., 1986; Mugrauer et al., 1988; Schmid et al., 1989; Morello et al., 1989; Mugrauer and Ekblom, 1991; Farquharson et al., 1992; Morgenbesser et al., 1995). During embryonic development, however, relationship between *c-myc* induction and cell proliferation appears to be cell/tissue/species-specific. A correlation between *c-myc* induction and cell proliferation was seen in mesoderm-derived but not ectoderm- and endoderm-derived structures (Pfiefer-Ohlsson et al., 1985; King et al., 1986; Downs et al., 1989; Schmid et al., 1989; Vandenbunder, et al., 1989; Hirvonen et al., 1990; Lemaitre et al., 1995). A reduced *c-myc* expression was observed in embryonic mouse (Morgenbesser et al., 1995) but not in chick lens cells (Nath et al., 1987; Harris et al., 1992). The *c-myc* proto-oncogene has also been implicated as an apoptosis promoting gene under conditions of restricted cell proliferation (Evan et al., 1992; Pakham and Cleveland, 1994). It has been suggested that during development, apoptosis is a physiological activity of c-Myc protein and is normally inhibited by growth factors or by expression of survival genes such as bcl-2 (Amati and Land, 1994; Harrington et al., 1994). As analyzed in the previous paragraphs, Myc protein functions as a sequence specific transcription factor that governs the regulation of target genes involved in various biological processes (Torres et al., 1992). Indeed, C-Myc protein is required for embryonic survival (Davis et al., 1993).

PURPOSE OF THE STUDY

One the major issues concerning the developmental biology of the secondary palate is how extracellular factors regulate various biological events such as cell proliferation, ECM synthesis, epithelial-mesenchymal interaction, and programmed cell death/cell transformation, during morphogenesis of the secondary palate. Previous studies have identified the involvement of several growth factors, including EGF, FGF, IGF, PDGF, and $TGF\beta_1$ in regulation of proliferation of embryonic palate mesenchymal cells. However, the mechanisms by which these growth factors regulate proliferation of palate mesenchymal cells are not known. The information is of significance because studies on the development of normal palate as well as teratogen-induced cleft palate have led to the concept that cell proliferation is one of the crucial biological events for advancement of palate morphogenesis. Hence, the present study was undertaken to (1) examine the effects of serum, EGF, $TGF\beta_1$, and their combination on DNA synthesis and proliferation of embryonic hamster palate mesenchymal cells (HPMC); and (2) the effects of serum, growth factors, and/or their combination on the expression of growth-related immediate early genes (*c-fos*, *c-jun*, and *c-myc*) in HPMC.

MATERIALS AND METHODS

Animal Maintenance and Breeding

Golden Syrian hamsters were used in this study. Male and female animals (100±10gm), 6-8 weeks old, were caged individually and acclimatized for a minimum of one week in an atmosphere of 50±5% humidity, 24±1°C temperature, and alternating cycles of light (6.00p.m. to 6.00a.m.) and dark. The food and water were available *ad libitum.* The animals were mated by placing one male and one female in a plastic cage. The male and female were allowed to mate from 7.00a.m. to 9.00a.m.. The midpoint of the mating period, 8.00a.m., was taken as the beginning of day 0 of gestation.

Collection of Embryonic Palatal Tissue

To collect embryonic tissue for cell culture, all the procedures were carried out in a sterile environment. On day 11:00 of gestation, the pregnant females were anesthetized by an intraperitoneal injection of 0.2 ml Sodium Pentobarbital (65mg/ml). The embryos from each female were collected and rinsed in a sterile 60mm culture plate containing 3 ml of Dulbecco's Modified Eagle Medium [DMEM (high glucose); Gibco/BRL Cat. No. 23700-040]. The palatal shelves were dissected using an Olympus dissecting microscope (6.5X magnification) and collected in a 60mm culture plate containing calcium magnesium-free/phosphate buffered saline (CMF/PBS) on ice.

Preparation of Primary Culture of Hamster Palate Mesenchymal Cells

To establish a primary culture of embryonic hamster palate mesenchymal cells (HPMC), the dissected palatal shelves were first rinsed in sterile ice-cold (0-4°C) CMF/PBS. The palates were then pooled, and minced thoroughly using a razor blade, and digested by incubation in 3ml of trypsin/EDTA solution [0.025% trypsin/0.27mM EDTA (Gibco/BRL; Cat. No. 610-5305AG) in CMF/PBS] in a sterile 15ml polypropylene tube (Fisher Scientific; Cat. No.14-956) at 37°C for 10 minutes. During the incubation, the

tube was gently shaken continuously. Subsequent to the digestion, the tissue homogenate was centrifuged at 1,000 rpm (90g) for 5 minutes at room temperature to pellet the cells. To inhibit the action of residual trypsin, the pellet was suspended and washed in 6 ml of ice-cold complete media [CM; DMEM (supplemented with 1mM sodium pyruvate, 44mM sodium bicarbonate and antibiotics: 60mg/l penicillin and 100 mg/l streptomycin) + 10% Fetal Calf Serum (FCS; Gibco/BRL, Cat. No. 26140-038)]. The cell suspension was then centrifuged at 600 rpm (33g) for 15 minutes at room temperature. The supernatant was discarded and the cells were re-suspended in 3ml CM. In order to determine the total number of cells in the suspension, trypan blue exclusion method was used; 50 µl of suspension was mixed with 40 µl of 0.2% trypan blue and 410 µl of CM, and vortexed. One drop of the mixture was placed on the hemocytometer and the cells were counted. The total cell number of cells in the suspension was calculated as the average number of cells/ hemocytometer grid x 10 (dilution factor, i.e., 50µl of cell suspension in 500µl of mixture) x 10^4 (conversion factor for hemocytometer grid to determine the number of cells per ml) x 3ml (total volume of suspension). Cells were then suspended in an appropriate volume of CM to obtain a density of 2.5 x 10^5 cells/ml and seeded into sterile plastic culture plates (Falcon, Cat. No. 3001). Cultures were maintained at 37°C with 5% CO_2 and 100% relative humidity. Media was changed on day 1 of plating, and every second day thereafter.

Growth Factor Treatment and Proliferation of HPMC in Primary Culture

In order to analyze the effects of serum and different growth factors on the rate of proliferation of HPMC, the cultured cells were maintained in CM for three days post-plating. They were then rinsed 3 times with 1ml serum-free DMEM, and maintained in serum-free DMEM for 24 hours for synchronization. At the end of the synchronization period, the cells were again rinsed three times with 1ml serum-free DMEM.

To analyze the effect of different concentrations of serum on HPMC proliferation, the cells were treated with, and subsequently maintained in DMEM containing 1%, 2.5%, 5%,

and 10% fetal calf serum for the length of the study. To examine the effects of co-treatment of growth factors on proliferation of HPMC, serum-starved cells were treated with either EGF (20ng/ml; GIBCO; Cat. No.3247SA), or TGFβ_1 (10ng/ml; Sigma Chemicals; Cat. No. T-1654), or with both EGF+TGFβ_1 for 24 hours. 20ng/ml EGF and 10ng/ml TGFβ_1 are the optimum dosages required to affect embryonic palate mesenchymal cells (D'Angelo and Greene, 1991; Shah et al., unpublished data)

In a separate experiment, following serum-starvation, cells were pre-treated with TGFβ_1 for 30 minutes. Subsequently, the TGFβ_1-containing DMEM was removed, and the plates were rinsed 3 times with serum-less DMEM. Cells were then treated with DMEM containing 2.5% serum, EGF, or EGF+2.5% serum (a time-course study indicated that 30 minutes TGFβ_1 was sufficient to inhibit DNA synthesis in HPMC).

After 24 hours, the plates were washed three times with fresh serum-free DMEM. Subsequently, the cells were maintained in DMEM containing 10% FCS. Growth-curves for serum treated and growth factor treated cells were obtained by counting the cells on days 0, 1, 3, 5, and 7 post-treatment.

To count the cells, the media from each plate was discarded, 1 ml of trypsin (1mg/ml) solution was added to each plate, and the plates were incubated in the water-bath at 37°C for 3-5 minutes. Subsequently, the cells were detached by repeated gentle pipetting with a pasteur pipette and then transferred into a glass culture tube (Fisher Scientific; Cat. No. 14-961-26) containing 0.5 ml of CM. Additional 0.5 ml aliquot of fresh CM was added to each plate and the remaining attached cells were scraped off the plate using a plastic scraper "Cell Lifter" (COSTAR, Cat. No.3008). The cells with CM from the plate were added to the suspension in the glass culture tube. After pipetting up and down a few times, 95 µl of cell-suspension and 5 µl of 0.2% trypan blue were mixed and vortexed. One drop of suspension was placed on the hemocytometer and the live cells were counted. The total number of the cells was determined as the average cell number on hemocytometer grid x 10^4 (conversion factor for hemocytometer grid to determine number of cells per ml) x 2 ml

(total volume of suspension). At each time, at least three plates were counted to determine the mean of one experiment. Each experiment was repeated at least three times. The mean and standard deviation of the mean were determined. For statistical analysis, 2-tailed student t-test was used (Zar, 1984).

Growth Factor Treatment and DNA Synthesis in HPMC in Primary Culture

HPMC were seeded in CM in 24 well culture plates (Falcon; Cat. No. 3047) at a density of 100,000 cells per well. On day 3 post-plating, cells were thoroughly rinsed three times with serum-free DMEM and then starved in serum-free DMEM for 24 hours. Subsequently, the cells were treated for 24 hours with 0.5 ml of the appropriate conditioned media, i.e., DMEM alone (untreated control) or DMEM containing: 2.5% serum, EGF (20ng/ml), $TGF\beta_1$ (10ng/ml), EGF+$TGF\beta_1$, EGF+2.5% serum, $TGF\beta_1$+2.5% serum, or EGF+TGFβ+2.5% serum.

For the $TGF\beta_1$ pre-treatment time course study, following 24 hour serum-starvation, HPMC were pre-treated with DMEM alone, or DMEM containing $TGF\beta_1$ for 5, 10, 20, 30 minutes or 1, 2, 4, or 6 hours. On completion of the pre-treatment, the cultures were rinsed three times with serum-less DMEM, and then treated with DMEM containing 2.5% serum, EGF, or EGF+2.5% serum for 24 hours.

At the end of the growth factor treatment, 1μCi/ml ^{3}H-thymidine (ICN; Cat. No.2404305) was added to the cultures for 3 hours. The media was then removed and the wells were rinsed three times with 0.5 ml of CMF/PBS. The HPMC in the wells were fixed by addition of 0.5 ml of 5% trichloroacetic acid (TCA) at 0-4°C for 30 minutes. Next, the TCA was removed, and the wells were rinsed three times with ice-cold TCA. To each well, 0.2 ml of 0.1M NaOH was added and the cells were incubated at 50°C for 1 hour to dissolve the DNA. The content of the wells were spotted on glass microfiber filters (Whatman; Cat. No. 1827-866), air dried overnight, and then transferred into 6 ml plastic scintillation

vials with 2 ml of scintillation fluid and counted for radioactivity in a Wallac 1410 Scintillation Counter (LKB). The radioactivity of the samples, indicative of ^{3}H-thymidine uptake, was measured as DPM, and the data were plotted for average DPM/well. Three wells were designated for each experiment and each experiment was repeated three times. Student t-test was used for statistical analysis of the data.

RNA Extraction from Primary Culture of HPMC

To collect RNA from serum- and growth factor-treated cells, the HPMC were seeded at a density of 500,000 cells/plate in 60mm culture plates (Falcon; Cat. No. 3002). On day 12 of culture, when the cells were still in pre-confluent phase of growth, the HPMC were rinsed with DMEM and serum-starved for 24 hours for synchronization. The cells were then treated with the appropriately conditioned media (serum and/or growth factor) for 5, 10, 15, or 30 minutes, or 1, 2, 6, 12, or 24 hours for the *c-fos* expression, and for 15 or 30 minutes, or 1,2, or 6 hours for *c-jun* and *c-myc* expression. To collect sufficient amount of total RNA for Northern blotting (20-30μg), 4-5 plates were used for each time point. At the end of the treatment period, the plates were rinsed three times with sterile CMF/PBS, and all the excess liquid was removed. The RNA was isolated by using Trizol reagent [(Gibco/BRL, Cat. No. 15596; Trizol reagent is a monophasic solution of phenol and guanidine isothiocyanate, which is used for single step RNA isolation (Chomczynski and Sacchi, 1987)]. HPMC were lysed directly in culture plates by adding 0.8 ml of Trizol reagent to a 60mm culture plate, and repeated pipetting. Each plate was scraped with a sterile plastic scraper to collect the lysate from the surface. The lysed sample was then transferred to the next plate in the same group, and the procedure was repeated until the RNA from all the plates in the group were collected in a sterile 1.5ml eppendorf tube. Next, the samples were incubated at room temperature for 5 minutes to permit the complete dissociation of nucleoprotein complexes. Subsequently, 0.2ml of chloroform was added to each sample. The tubes were shaken vigorously by hand for 15 seconds and incubated at

room temperature for 2-3 minutes. The samples were then centrifuged at 12,000g for 15 minutes at 2-8°C. Following centrifugation, the mixture separated into three phases: a lower red, phenol-chloroform phase, a white interphase of DNA, and an upper colorless aqueous phase. RNA remains exclusively in the aqueous phase. The volume of the aqueous phase was usually about 60% of the Trizol reagent used for cell lysis. The aqueous phase was gently transferred into a fresh eppendorf tube. The RNA from the aqueous phase was precipitated by adding 0.5ml of isopropyl alcohol. The samples were then incubated at room temperature for 10 minutes and centrifuged at 12,000g for 10 minutes at 2-8°C. The RNA precipitate often formed a gel-like pellet on the side and bottom of the tube. After centrifugation, the supernatant was removed and the pellet was re-suspended with 1ml of 75% ethanol for a rinse. The sample was vortexed and centrifuged at 7,500g for 5 minutes at 2-8°C. The alcohol was discarded and the RNA pellet was air dried for 15-20 minutes. The RNA pellet was then dissolved in 0.2ml of RNase-free water by passing the solution a few times through a pipette tip (RNase-free water was prepared by adding 0.5ml of diethylpyrocarbonate [(DEPC); Sigma, Cat. No.D-5758] to 1L of sterile water overnight and autoclaving it). Subsequently, the total amount of RNA in the samples was determined. A 0.5µl aliquot of the RNA samples was added to 0.495ml of RNase-free water and the optical density (OD) was measured using a Beckman DU-600 spectrophotometer. To calculate the total amount of RNA in the samples, the following formula was used:

OD at 260nm X 100 (dilution factor)x 40 (conversion factor from OD to µg; a solution that has an OD of 1 contains approximately 40µg of RNA per ml)X 0.2ml (total volume of the RNA samples) = total amount of RNA in sample (µg).

In order to reconstitute the RNA samples to a desired concentration, the RNA in the samples were precipitated by adding 25µl of 3M sodium acetate and 0.7ml of 100% ethanol to the samples and left at -20°C over night. Subsequently, the samples were centrifuged at 12,000g for 15 minutes, and the supernatants were discarded. The samples were air-dried

for 15-20 minutes, dissolved in the proper volume of RNase-free water at a concentration of 3 or 5μg/μl, and stored at -70°C.

Northern Blotting

a) Electrophoresis of RNA Samples

For electrophoresis of the RNA samples, 1% agarose gel containing 0.66M Formaldehyde and 1X 3-(N-morpholinol) propanesulfonic acid (MOPS) was prepared. In detail, 1g of agarose (Gibco/BRL, Cat. No.5510UA) was melted in 100ml of RNase-free water and cooled to 60°C. Subsequently, 10ml of 10X MOPS (Sigma, Cat. No.M1254) and 2.5ml of deionized formaldehyde [Fisher Scientific, Cat. No.F79-500; (Formaldehyde and formamide were deionized by adding 1g of AG 501-X8 mixed-bed resin (Bio-Rad, Cat. No.142-6424) to 20 ml of each solution and stirring on a vortex for 1 hour)].

To prepare the RNA samples for loading on the gel, they were denatured in 50% deionized formamide, 2.2M formaldehyde, and 20mM MOPS pH 7.0. In detail, 5μl of each RNA sample was mixed (prepared at 3 or 5μg/μl) with a sample buffer containing 6μl of deionized formamide (Fisher Scientific, Cat. No.BP227-500), 2μl of deionized formaldehyde and 0.6μl of 10X MOPS for each sample (total amount RNA loaded on the agarose gels was 15μg for the initial studies (*c-fos*); however, since hybridization of *c-jun* and *c-myc* transcripts was not successful at this concentration of RNA, 25μg of RNA was loaded on the gels for further studies). The samples were incubated in a 65°C water bath for 15 minutes, and chilled on ice. They were subsequently centrifuged for 5 seconds to deposit all the liquid to the bottom of the eppendorf tube. To each sample, 1μl of loading dye (a mixture solution of 10% xylene cyanole and 10% bromophenol blue) and 1μl of ethidium bromide [(1mg/ml); Sigma, Cat. No.E8751] were added. As a molecular weight marker, a pre-stained 0.24-9.5 Kb RNA ladder (Gibco/BRL, Cat. No.15620-016) was used. The gel was casted in a horizontal electrophoresis gel box (Strategene, Cat. No.40043) inside a chemical hood, and was allowed to set for a minimum of 30 minutes at room temperature.

1X MOPS was used for running buffer and samples were sequentially loaded on the gel. The gel was then run at 80-100V for 1.5-2 hours.

b) Transfer of Denatured RNA to Nylon Membrane

After electrophoresis, the gel was washed in RNase-free water and immersed in 10X SSC for 45 minutes with gentle agitation to remove the formaldehyde before transfer and hence improve the transfer quality. Two methods were employed for transferring denatured RNA to nylon membrane: capillary elution and pressure blotting.

i) Capillary Blotting

For capillary elution blotting the method described by Sambrook et al (1989), in which the gel is placed in contact with nylon membrane to facilitate the transfer of RNA to the nylon membrane through an ascending flow of buffer, was used. To set the system, a glass plate support with a surface area well larger than the gel was placed inside a large baking dish (the height of the glass support should be taller than the depth of the dish). The dish was filled with 10X standard saline citrate [(SSC); 0.015 M sodium citrate and 0.15 M sodium chloride)] solution until the level of solution reached three-quarter of the height of the support. A long strip of 3MM Whatman paper was cut, briefly soaked in 10X SSC, and placed over the glass support into the reservoir- the paper was wider than the gel and hung over the support to the reservoir. The nylon membrane was cut exactly to the size of the gel and soaked in 10X SSC for 2 minutes. The nylon membrane that was used in this study was Hybond-N (Amersham, Cat. No.RPN.303N), which has high sensitivity in RNA blotting and high physical strength that can endure several stripping and reprobing procedures. The gel was placed on the support in an inverted position so that it is centered on the wet 3MM paper. The wetted nylon membrane was placed on top of the gel. Two pieces of 3MM paper (cut exactly to the size of the gel) were wetted in 10X SSC and placed on top of the wet nylon membrane. A stack of paper towels (5-8 cm) was cut to the size of the gel and placed on the 3MM papers. A glass plate and a lead weight of approximately 0.75 kg was put on top of the

stack. The transfer was allowed to proceed for 16-20 hours. During the transfer, buffer is drawn from the reservoir and passes through the gel into the stack of paper towels. The nucleic acids elude from the gel by the moving stream of the buffer and deposit on the nylon membrane. The weight applied on the top of the paper towels provides a tight connection between the layers of material used in the transfer system.

ii) Pressure Blotting

The second transfer method was using a pressure blotter, PosiBlot 30-30 system (Strategene, Cat. No.400330-3). This method essentially resembles the capillary blotting, except that in this technique, pressure is exerted to a reservoir of buffer from the top, which eludes the RNA to the nylon membrane by a descending flow of the buffer. Similar to the capillary blotting technique, subsequent to electrophoresis, the gel is rinsed in 10X SSC for 45 minutes. Two pieces of 3MM Whatman papers and one piece of nylon membrane were cut to the size of the gel and soaked in 10X SSC for 2-3 minutes.

The apparatus is composed of: a box which acts as a buffer collection base, a plastic support, a membrane support pad, a PVC mask cut by at least 0.3 cm smaller on all four sides than the size of the nylon membrane, and a cellulose sponge that acts as a buffer reservoir. The apparatus was set up as described in the company's manual (Strategene, Cat. No.400330-3).

The sponge was soaked in 10X SSC for about 10 minutes prior to the assembling of the apparatus. A wetted 3MM Whatman paper was placed on the center of the support, followed by the wetted nylon membrane and the gel. The mask was fixed on top of the membrane in a way that the upper edge of the rectangular window lined up below the rows of wells and the other edges overlapped the gel. A wetted 3MM whatman paper was laid on the gel and all the trapped air bubbles were pushed out. The soaked sponge was gently laid over the gel assembly, the lid was closed and the latches were tightly fastened to prevent any air leakage. The pressure control station was adjusted to 90-100Hg, and the connector hose

was attached to the blotter inlet port. The blotting was done in 60-75 minutes, the completion of the transfer was checked by using a hand-held UV illuminator.

After the allotted blotting time for either method, using a hand-held UV illuminator, the position of the wells and RNA ladder was pencil marked on the membrane. The nylon membrane was then removed from the device and placed on a clean 3MM Whatman paper to allow the excess buffer to be absorbed. Once the membrane was free of standing liquid, but still damp, it was wrapped in saran wrap and exposed to UV light on a 312nm transilluminator for about 3 minutes to fix the RNA on the membranes. The membrane was then washed in 1% SDS for 5 minutes, sealed in plastic bag and stored in -20°C freezer.

c) Hybridization of Radio-labelled Probes to Immobilized RNA Samples

For hybridization, the method by Sambrook et al (1989) was employed. In brief, the membranes were submerged in 6X standard saline phosphate/EDTA [(SSPE); 0.015M sodium citrate, 0.2M sodium phosphate, and 0.2M EDTA] for 2 minutes. Subsequently, the membranes were transferred to a RNase-free -sealable hybridization bag, which was filled with 10ml of pre-hybridization solution containing 50% formamide, 2X Denhardt's solution [2% of each of Ficoll (type 400 Pharmecia), polyvinylpyrrolidone, and bovine serum albumin], 5X SSPE, and 0.1% SDS for 5-8 hours at 42°C. Subsequently, for hybridization, 10μCi ^{32}P-dCTP oligolabelled DNA probe (*c-fos, c-jun, c-myc*, or GAPDH; see below) was added to the pre-hybridization solution. The membranes were hybridized over night (16-18 hours) at 42°C.

On completion of hybridization, membranes were washed as follows to remove the unbound DNA probes. When hybridized with GAPDH or *c-fos,* membranes were washed once with 1XSSC/0.1%SDS at room temperature for 30 minutes, then twice with 0.2X SSC/0.1% SDS at 55°C for 45 minutes, and finally, once with a high-stringency solution of 0.1X SSC/0.1% SDS at 55°C for 30 minutes. Lower stringency conditions were used when membranes were hybridized with *c-jun*, or *c-myc*: membranes were first washed with 2X

SSC/0.1% SDS, then twice with 1XSSC/0.1% SDS at 55°C for 45 minutes, and finally with 0.5%X SSC/0.1% SDS at 55°C for 30 minutes. The membranes were then dried, wrapped in Saran Wrap, and exposed to Crovex 4 X-ray film (Dupont, Cat. No. 100 NIF) with an intensifier screen at -70°C. The exposure time varied depending on the probe and the specific activity of the isotope used. Generally, the exposure time was 2-3 days for GAPDH, 3-4 days for *c-fos*, and 7-10 days for *c-jun* and *c-myc*. X-ray films were developed in an automatic developer machine. The autoradiograms were then scanned, using Ophoto program. Subsequently, the intensity of expression of the genes were quantified by performing densitometry analysis on the scanned images, using Image (NIH) program. The values obtained for *c-fos*, *c-jun*, and *c-myc* signals were corrected for the variations in amount of RNA loaded on agarose gels, using GAPDH signals.

When not examined, the membranes were wrapped in Saran Wrap, and kept at -20°C until the next hybridization. Prior to rehybridization with a new probe, the membranes were stripped using the method described by Sambrook et al (1989). Briefly, membranes were immersed in a solution of 50% formamide and 2X SSPE for 1 hour at 65°C. Subsequently, membranes were rinsed briefly with 0.1X SSPE at room temperature and dried. At regular intervals, stripped membranes were exposed to X-ray films to ensure that all the signals were removed.

d) Preparation of Radio-labelled Probes

The cDNA probes for *c-fos*, *c-jun*, and GAPDH were received as gifts from Dr. P. Rathana Swami (Bio-medical Research Center, University of British Columbia), and Dr. Wong (Dental School, Harvard University). The cDNA probe for *c-myc* was purchased from ONCOR company (Cat No. P2110; 3rd exon, Eco R1/CH4A excised 1.4kb fragment isolated from human Burkitt's lymphoma genomic library). Probes were radio-labelled using the Gibco/BRL random primer labelling system (Gibco/BRL; Cat. No. 18187-013). To prepare the probes for hybridization, 10ng of probe was dissolved in 13µl of RNase-free

water, and heat-denatured at 98°C for 5 minutes. Subsequently, 9µl of oligomix (a mixture of: 1µl of 0.5mM solution from each dATP, dGTP, and dTTP, and 6µl of random primers buffer mixture containing, 0.67M HEPES, 0.17M Tris-HCL, 17mM $MgCL_2$, 33mM 2-mercaptoethanol, 1.33mg/ml BSA, 18 OD_{260} units/ml oligonucleotide primers (hexameres), pH 6.0), 2.5µl of [α-^{32}P]-dCTP, and 0.5µl of Klenow fragment (large fragment of DNA polymerase I in 100mM potassium phosphate buffer (pH 7.0), 10mM 2-mercaptoethanol, 50% (v/v) glycerol) were added to the probe and incubated at room temperature for 3-5 hours. The probes were heat-denatured at 98°C for 5 minutes, prior to addition to the hybridization solution.

RESULTS

A) Effects of serum and growth factors on proliferation of hamster palate mesenchymal cells (HPMC)

1. Effects of different concentrations of serum on proliferation of HPMC

The data on the proliferation of HPMC following treatment with media (DMEM) containing 1%, 2.5%, 5%, and 10% serum are outlined in figure 9.

When HPMC were treated with 1% serum in the culture media the cell number declined by 65% between days 0 and 7 post treatment ($P<0.05$).

In the presence of 2.5% serum in the culture media, however, the cell number increased 2.4 fold between days 0 and 7 post-treatment ($P<0.005$).

When the concentration of serum in culture media was raised to 5% and 10%, the number of HPMC increased 4.3 fold and 5.6 fold, respectively, between days 0 and 7 post-treatment ($P<0.005$).

These results indicate that *in vitro* rate of proliferation of HPMC depends on concentration of serum in culture media. The data also shows that at least 2.5% serum is required in culture medium to sustain the survival and growth of HPMC.

2. Effects of growth factors EGF, TGFβ_1, and their combination on proliferation of HPMC

The data on the proliferation of HPMC, following 24 hour treatment with media (DMEM) alone or media containing 10% serum, EGF (20ng/ml), TGFβ_1(10ng/ml), or their combination (EGF+TGFβ_1) are presented in figure 10.

The serum-starved DMEM-treated HPMC showed a 2.9 fold increase in cell number between days 0 and 7 post-treatment. Treatment of HPMC with 10% serum or EGF (20ng/ml) increased the cell number 5.6 fold and 4.3 fold, respectively, between days 0

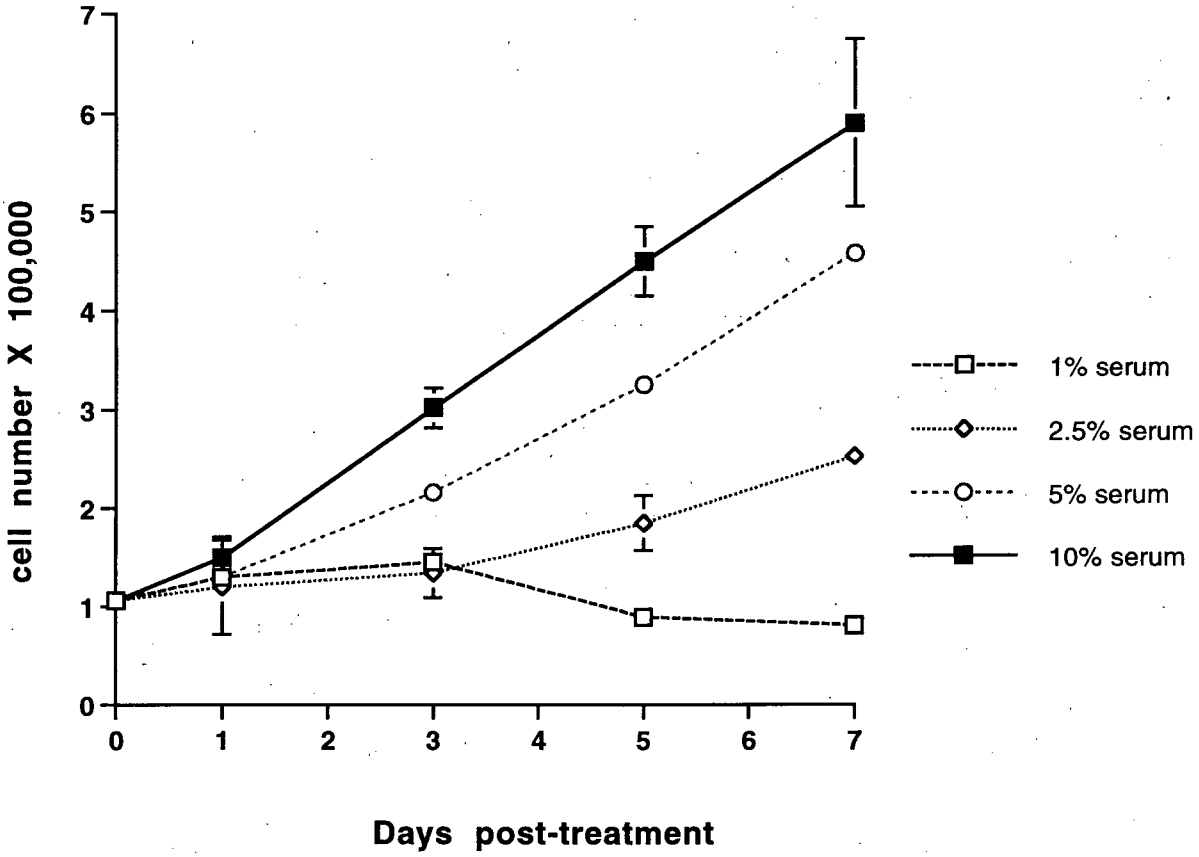


Figure 9. Effects of different concentrations of serum on HPMC proliferation. HPMC were grown in DMEM containing 10% serum for 3 days. Cells were then serum starved for 24 hours, and maintained in presence of media containing DMEM and 1%, 2.5%, 5%, or 10% of serum for 7 days. Media was changed every second day. Using trypan blue exclusion method, cells were counted on days 0, 1, 3, 5, and 7 post-treatment to obtain a growth curve.

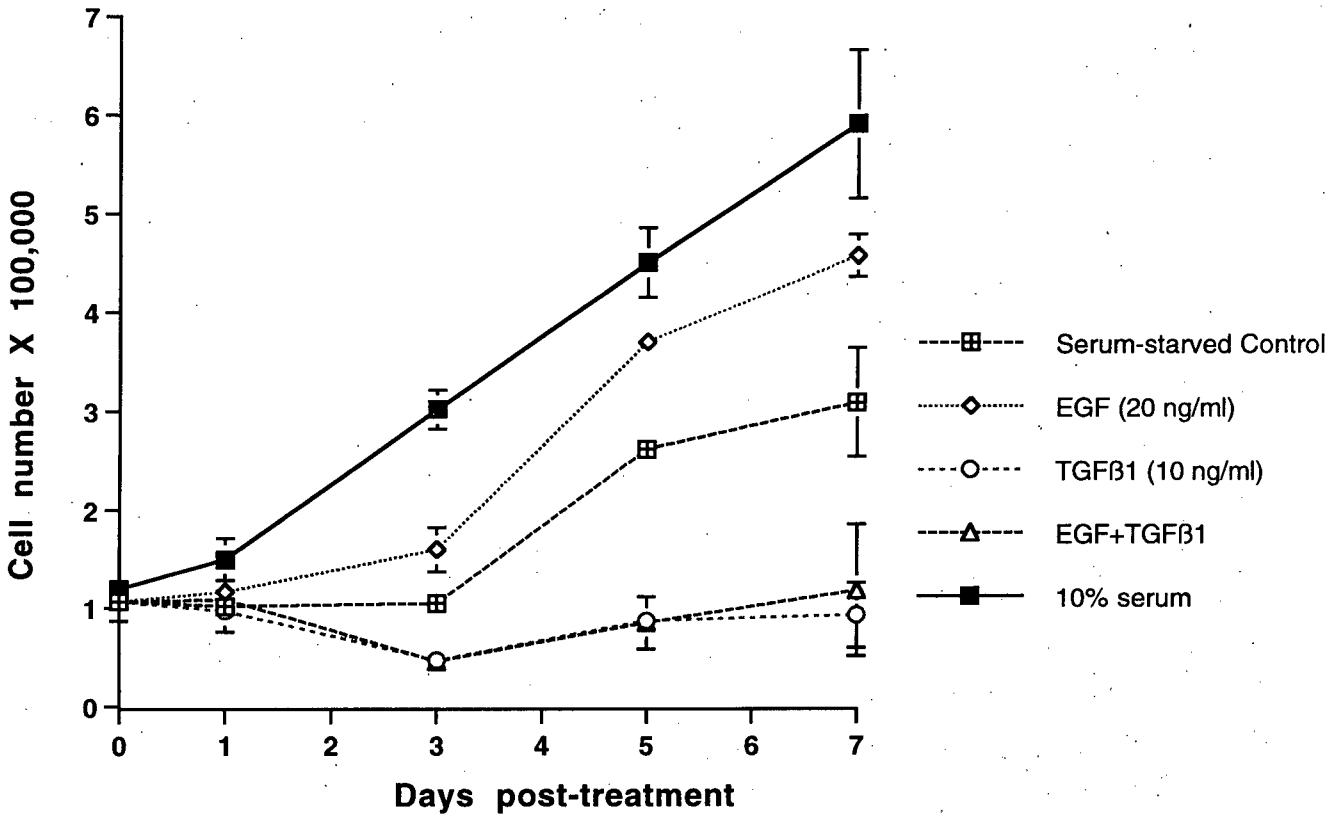


Figure 10. Effect of EGF, TGFβ_1, and their combination on HPMC proliferation. HPMC were serum starved for 24 hours, and treated with DMEM alone, or DMEM containing 10% serum, EGF, TGFβ_1, or EGF+TGFβ_1 for 24 hours. Subsequently, cells were maintained in media containing 10% serum for the duration of the culture period. Media was changed every second day. Using trypan blue exclusion method, cells were counted on days 0, 1, 3, 5, and 7 post-treatment to obtain a growth curve.

and 7 post-treatment ($P<0.001$). On the other hand, treatment of HPMC with $TGF\beta_1$ alone, or in combination with EGF did not affect the cell number ($P<0.05$).

In comparison to the serum-starved controls, EGF and 10% serum exposed cultures grew faster and were 1.5 and 2 folds higher in number on day 7 of treatment ($P<0.005$), whereas $TGF\beta_1$ and $EGF+TGF\beta_1$ treated ones were 70% and 60% less in number, respectively ($P<0.001$).

These observations suggest that EGF exerts mitogenic effects on HPMC, whereas $TGF\beta_1$ does not support proliferation of HPMC. Furthermore, when HPMC are co-treated with $TGF\beta_1$ and EGF, $TGF\beta_1$ overcomes the mitogenic effects of EGF by exerting its growth inhibitory effects on cells.

3. Effects of $TGF\beta_1$ pre-treatment on serum- and EGF-induced proliferation of HPMC

To further analyze the effect of interacting growth factors on proliferation of HPMC, cells were pre-treated with $TGF\beta_1$ for 30 minutes followed by 2.5% serum, EGF, or EGF+2.5% serum. The data on the proliferative behavior of serum- and/or EGF-treated HPMC following pre-treatment with $TGF\beta_1$, are shown in figure 11.

When HPMC were maintained in presence of 2.5% serum or 10% serum without $TGF\beta_1$ pre-treatment, they showed 2.3 and 5.2 fold increase in cell number, respectively, between day 0 and 7 post-treatment ($P<0.005$). When HPMC were, however, pre-treated with $TGF\beta_1$ for 30 minutes prior to treatment with 2.5% serum, EGF, or EGF+2.5% serum, the cell number did not change between day 0 and 7 post-treatment ($P<0.05$).

These data indicate that 30 minutes of $TGF\beta_1$ pre-treatment is sufficient to inhibit cell proliferation in HPMC, and to overcome the mitogenic effects of serum and EGF in these cells.

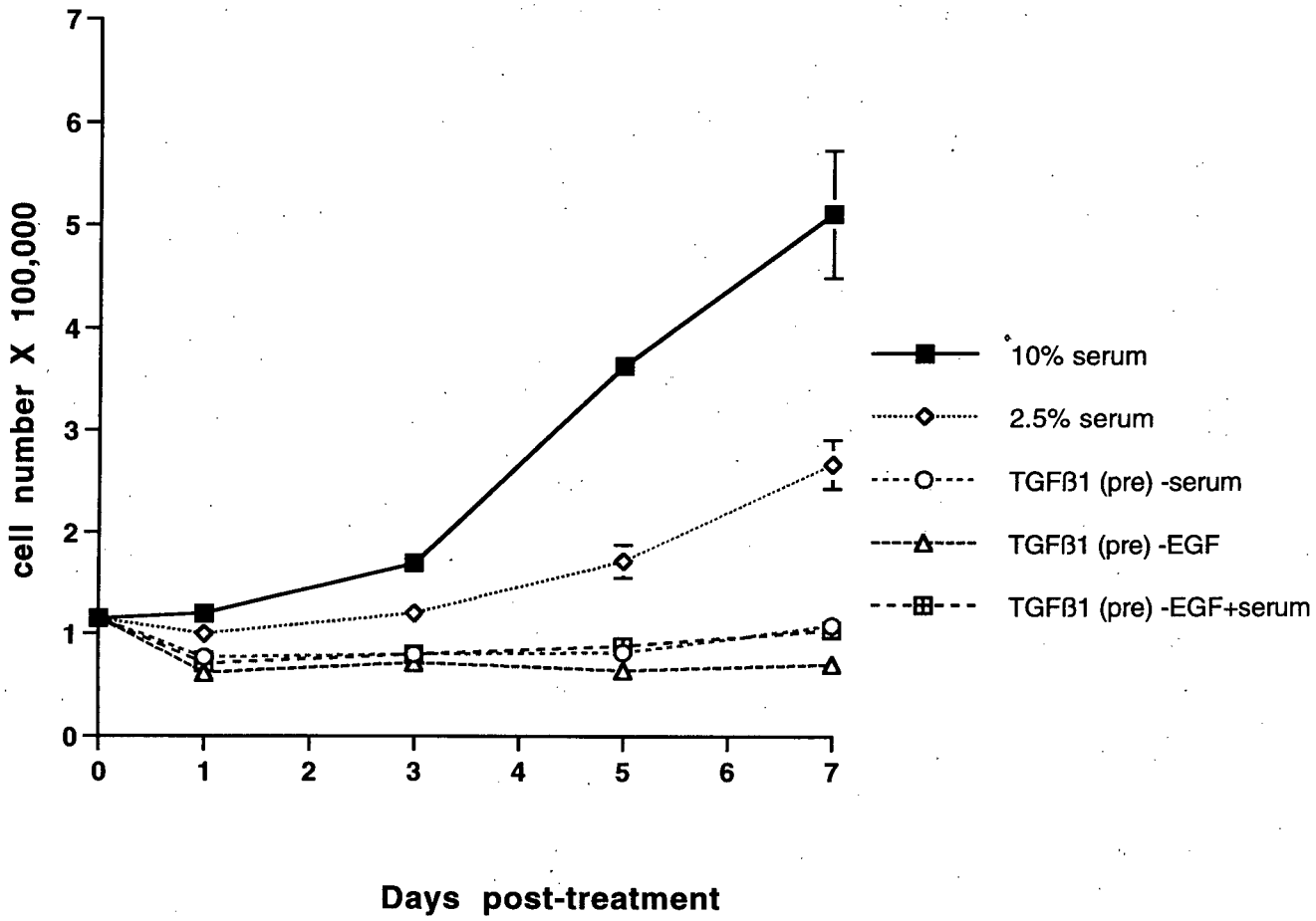


Figure 11. Effect of TGFβ_1 pre-treatment on serum- and/or EGF-induced HPMC proliferation. HPMC were serum starved for 24 hours, and treated with TGFβ_1 for 30 minutes. Cells were then rinsed with serum-less media, and treated with 2.5% serum, EGF, or EGF+2.5% serum for 24 hours. Subsequently, HPMC were maintained in media containing 10% serum for the duration of culture period. Media was changed every second day. Using trypan blue exclusion method, cells were counted on days 0, 1, 3, 5, and 7 post-treatment to obtain a growth curve.

B) Effects of serum and growth factors on DNA synthesis in HPMC

1. Effects of serum (2.5%) and growth factors EGF, TGFβ_1 and their combination on DNA synthesis of HPMC

The data on DNA synthesis in HPMC following 24 hour treatment with media containing 2.5% serum, EGF, TGFβ_1, or their combinations are outlined in figure 12.

Following treatment with 2.5% serum, the DNA synthesis in HPMC (as measured by ^{3}H-thymidine incorporation) was 3.3 fold higher than untreated serum-starved group ($P<0.001$).

Treatment of HPMC with EGF alone did not affect DNA synthesis. When cells were treated with EGF in the presence of 2.5% serum, however, DNA synthesis was increased by 1.6 fold in comparison to the 2.5% serum-treated control ($P<0.005$). These data suggest that presence of serum is necessary to support the EGF-induced DNA synthesis in HPMC.

On the other hand, treatment of HPMC with TGFβ_1 alone or with TGFβ_1 in the presence of 2.5% serum reduced the DNA synthesis by 70-80%, in comparison to serum-starved or 2.5% serum treated control ($P<0.005$). Thus, the data suggest that TGFβ_1 inhibits DNA synthesis both in presence or absence of serum.

Similarly, co-treatment of HPMC with both EGF and TGFβ_1, in the presence/absence of serum resulted in a decrease in DNA synthesis by 70-72% in comparison to the controls ($P<0.005$).

These results suggest that EGF (in presence of serum) supports, whereas TGFβ_1 does not support DNA synthesis in HPMC. Furthermore, presence or absence of serum modulates the effect of EGF, but not TGFβ_1 on DNA synthesis.

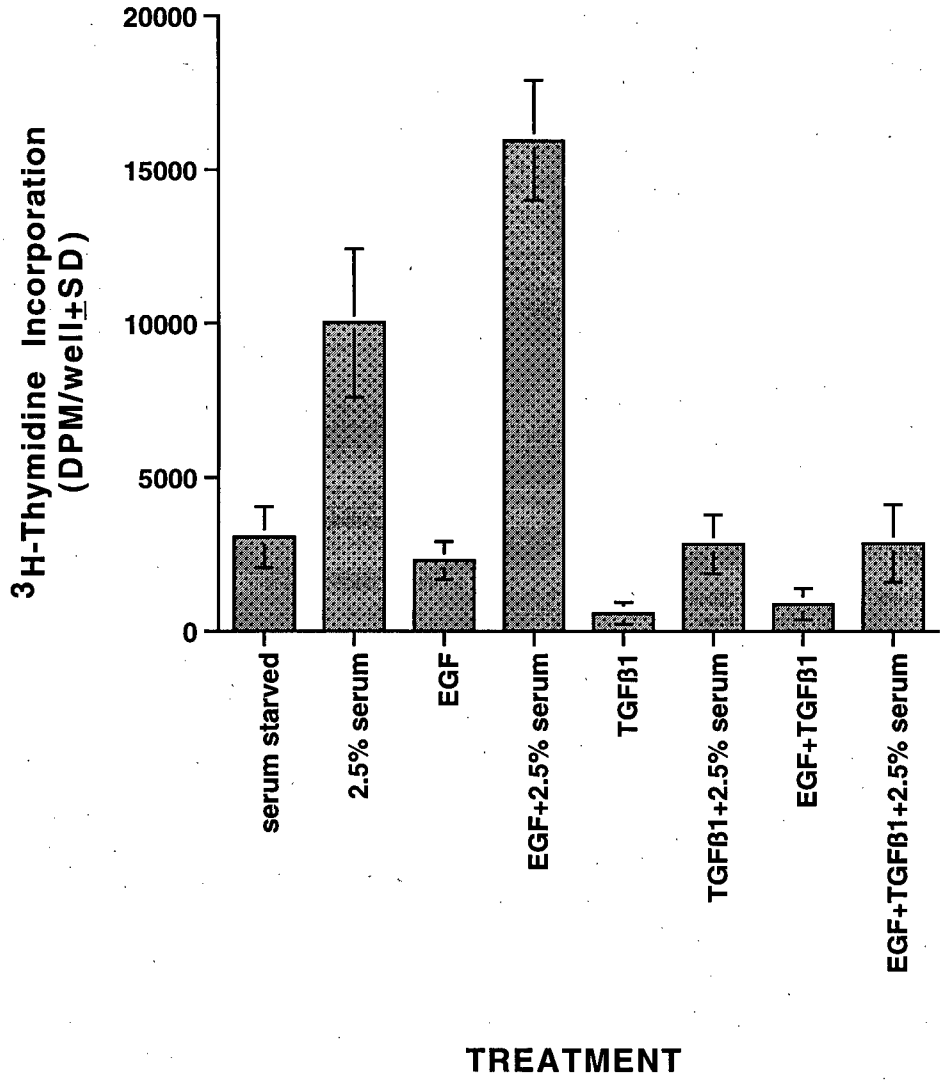


Figure 12. Effects of serum, and growth factors on DNA synthesis in HPMC. Cells were serum starved for 24 hours, and treated with DMEM alone, or DMEM containing 2.5% serum, EGF, EGF+2.5% serum, TGFβ_1, TGFβ_1+2.5% serum, EGF+TGFβ_1, or EGF+TGFβ_1+2.5% serum. ^{3}H-thymidine (1μCi/ml) was added to the culture media, during the last 3 hour of serum/growth factor treatment. The incorporation of ^{3}H-thymidine into acid-insoluble material was measured by liquid scintillation counting. Values are presented as DPM means ± SD.

2. Effects of TGFβ_1 pre-treatment on serum- and EGF-induced DNA synthesis of HPMC

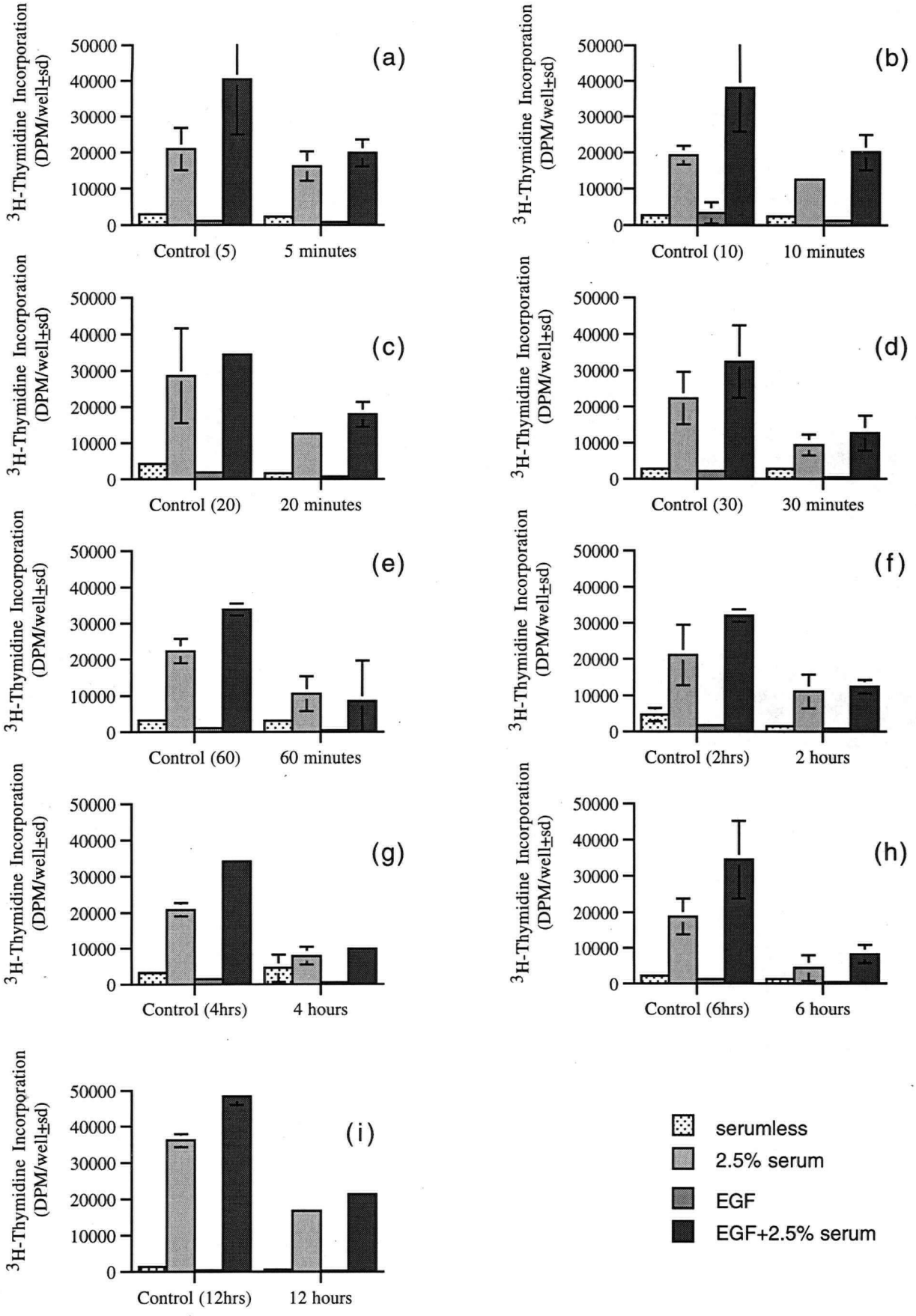
To further analyze the effects of interacting growth factors on DNA synthesis of HPMC, cells were pre-treated with TGFβ_1. A time course study was performed to determine the minimum optimum time required for TGFβ_1 to exert its inhibitory effects on the EGF/serum-induced DNA synthesis in HPMC (figure 13a-i). In comparison to controls (TGFβ_1 un-treated), pre-treatment of HPMC with TGFβ_1 for 5, 10, or 20 minutes, followed by treatment with 2.5% serum, EGF, or EGF+2.5% serum did not affect DNA synthesis significantly ($P<0.05$; figure 13a-c).

On the other hand, following 30 minutes pre-treatment of HPMC with TGFβ_1, DNA synthesis in 2.5% serum-, EGF-, and EGF+2.5% serum-treated cells was reduced significantly (58%, 81%, and 61%), respectively, in comparison to the cells without TGFβ_1 pre-treatment ($P<0.05$, figure 13d).

Extending the duration of TGFβ_1 pre-treatment of HPMC for up to 12 hour also resulted in significantly decreased DNA synthesis as compared to the controls ($P<0.05$; figure 13e-i).

These results show that pre-treatment of HPMC with TGFβ_1 for at least 30 minutes is sufficient to inhibit the serum- or EGF- induced DNA synthesis in HPMC.

Figure 13. Time course study showing the effects of TGFβ_1 pre-treatment on serum- and EGF-induced DNA synthesis in HPMC. Cells were serum-starved for 24 hours, and treated with DMEM alone, or DMEM containing TGFβ_1 for 5, 10, 20, or 30 minutes, and 1, 2, 4, 6, and 12 hours. Control cells were treated with DMEM alone for the same durations. Subsequently, cells were treated with 2.5% serum, EGF, or EGF+2.5% serum for 24 hours alone. ^{3}H-thymidine (1μCi/ml) was added to the culture media during the last 3 hour of treatment. The incorporation of ^{3}H-thymidine into acid-insoluble material was measured by scintillation counting. Values are presented as DPM means $\pm$ SD.


^{3}H-Thymidine Incorporation (DPM/well±sd)
50000
40000
30000
20000
10000
0
(a)
Control (5)
5 minutes
(b)
Control (10)
10 minutes
(c)
Control (20)
20 minutes
(d)
Control (30)
30 minutes
(e)
Control (60)
60 minutes
(f)
Control (2hrs)
2 hours
(g)
Control (4hrs)
4 hours
(h)
Control (6hrs)
6 hours
(i)
Control (12hrs)
12 hours
serumless
2.5% serum
EGF
EGF+2.5% serum

C) Northern blot analysis of effects of serum and growth factors (EGF and TGFβ_1) on the expression of immediate early genes *c-fos*, *c-jun*, and *c-myc* in HPMC

During the course of this study, the physiological state of the cells and the variations in the amount of RNA loaded on agarose gels were controlled by measuring the expression of GAPDH, a house-keeping gene, which encodes an enzyme in the glycolytic pathway, and its mRNA expression is not affected by serum and growth factors.

1. Effects of serum:

1a. Effects of different concentrations of serum on expression of *c-fos* mRNA

The autoradiograms showing the expression of *c-fos* in HPMC following treatment with media containing 2.5% and 10% serum are depicted in figure 14.

The serum-starved, DMEM-treated HPMC did not show *c-fos* expression (lane C, of figures 14a and 14b). Treatment of HPMC with 2.5% serum showed a signal for *c-fos* expression at 30 minutes. The signal persisted until 1 hour, albeit at a lower level, and subsequently disappeared. Exposure of HPMC to 10% serum, on the other hand, depicted *c-fos* mRNA signal at 15 minutes, which gradually increased in intensity until 1 hour, and disappeared thereafter.

The data on densitometric analysis revealed that, following treatment of HPMC with 2.5% serum, the expression of *c-fos* mRNA decreased by 63% between 30 minutes and 1 hour. In contrast, after exposure of HPMC with 10% serum, the expression of *c-fos* mRNA increased approximately 4 fold between 15 minutes and 1 hour.

These data indicate that the serum concentration in the culture media modulates *c-fos* gene expression; higher concentration of serum results in early and increasingly intense expression of *c-fos* mRNA.

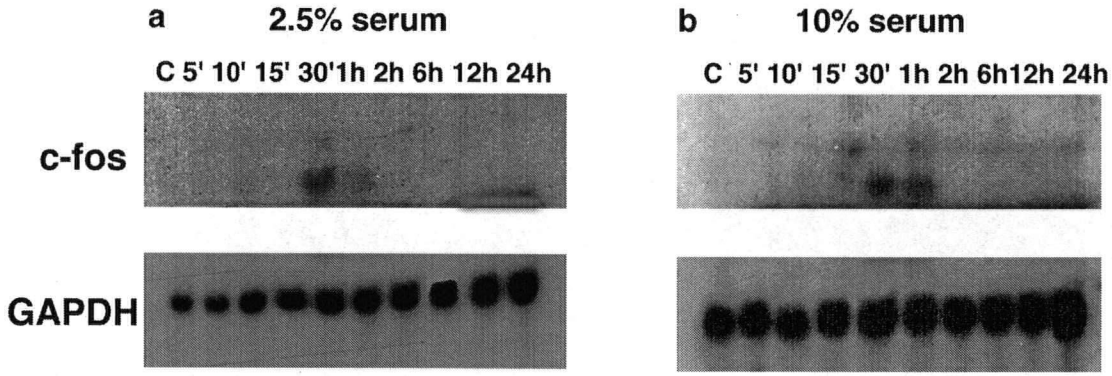


Figure 14. Northern blot analysis of effects of 2.5%(a) and 10%(b) serum on expression of *c-fos* mRNA in HPMC. HPMC were serum-starved for 24 hours, and then treated with DMEM containing 2.5% or 10% serum for 5, 10, 15, or 30 minutes, or 1, 2, 6, 12, or 24 hours. The controls were treated with DMEM alone. To perform Northern analysis, RNA was extracted from HPMC. An aliquot of 15 μg RNA was loaded per lane, separated on 1.2% agarose gel, transferred onto a nylon membrane, which was then hybridized using ^{32}P-labelled probes. *c-fos* expression was detected at 2.2kb. GAPDH gene expression (1.8kb) was used as a control.

1b. Effects of different concentrations of serum on expression of *c-jun* mRNA

The autoradiograms showing the expression of *c-jun* in HPMC following treatment with media containing 2.5% and 10% serum are shown in figure 15.

Expression of *c-jun* mRNA was not detectable in HPMC treated with DMEM alone or 2.5% serum (lane C, figures 15a and 15b; figure 15a). On the other hand, when cells were treated with 10% serum, *c-jun* mRNA was expressed within 15 minutes. Subsequently, the intensity of *c-jun* signal increased until 1 hour and then gradually declined by 6 hours post-treatment (figure 15b).

The densitometry analysis of HPMC exposed to 10% serum showed that expression of *c-jun* mRNA signal intensified 2.4 fold by 1 hour, but decreased thereafter.

These data suggest that serum concentration in the culture media modulates transcription of *c-jun*: whereas 2.5% serum is not sufficient to stimulate *c-jun* expression in HPMC, 10% serum rapidly triggers *c-jun* expression in HPMC.

1c. Effects of different concentrations of serum on expression of *c-myc* mRNA

The autoradiograms showing the expression of *c-myc* in HPMC following treatment with media containing 2.5% and 10% serum are presented in figure 16.

Serum-starved HPMC treated with DMEM alone do not show *c-myc* expression (lane C, figures 16a and 16b). HPMC treated with 2.5% serum expressed *c-myc* within 30 minutes, albeit at low levels. Subsequently, *c-myc* mRNA signal was intensified by 1 hour, but declined thereafter by 6 hours (figure 16a). When treated with 10% serum, HPMC expressed *c-myc* mRNA within 1 hour. The signal intensity then reduced but persisted up to at least 6 hours (figure 16b).

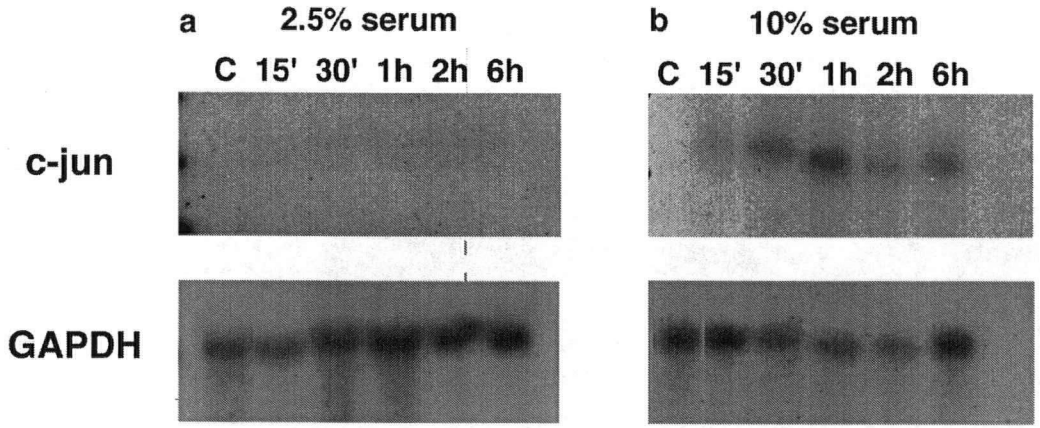


Figure 15. Northern blot analysis of effects of 2.5%(a) and 10%(b) serum on expression of *c-jun* mRNA in HPMC. HPMC were serum-starved for 24 hours, and then treated with DMEM containing 2.5% or 10% serum for 15, or 30 minutes, or 1, 2, or 6 hours. The controls were treated with DMEM alone. To perform Northern analysis, RNA was extracted from HPMC. An aliquot of 25 μg RNA was loaded per lane, separated on 1.2% agarose gel, transferred onto a nylon membrane, which was then hybridized using ^{32}P-labelled probes. *c-jun* expression was detected at 2.7kb. GAPDH gene expression (1.8kb) was used as a control.

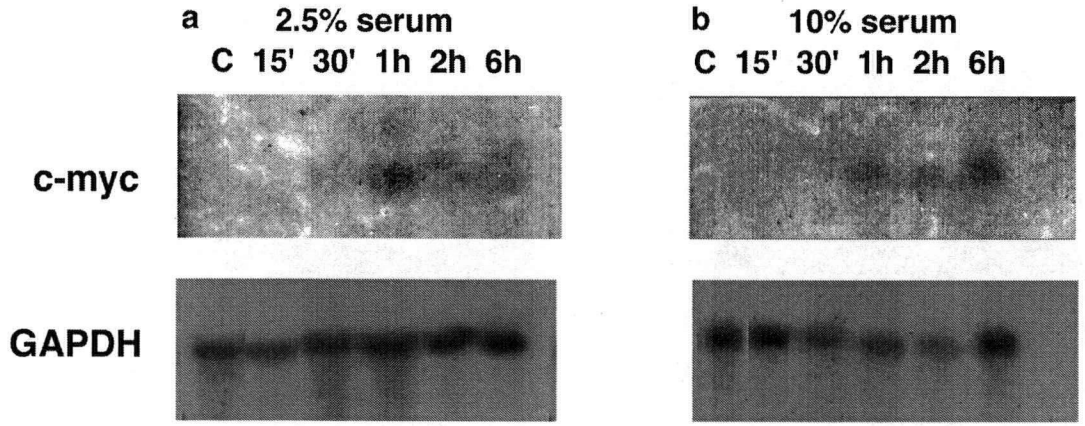


Figure 16. Northern blot analysis of effects of 2.5%(a), 10%(b), serum on expression of *c-myc* mRNA in HPMC. HPMC were serum-starved for 24 hours, and then treated with DMEM containing 2.5% or 10% serum for 15, or 30 minutes, or 1, 2, or 6 hours. The controls were treated with DMEM alone. To perform Northern analysis, RNA was extracted from HPMC. An aliquot of 25 μg RNA was loaded per lane, separated on 1.2% agarose gel, transferred onto a nylon membrane, which was then hybridized using ^{32}P-labelled probes. *c-myc* expression was detected at 2.3kb. GAPDH gene expression (1.8kb) was used as a control.

The densitometry observations indicated that in 2.5% serum-treated HPMC, the intensity of *c-myc* expression increased approximately 2 fold between 30 minutes and 1 hour. Subsequently, the signal decreased 31% by 2 hours, and remained unchanged at 6 hours post-treatment. Similarly, following treatment of HPMC with 10% serum expression of *c-myc* declined 33% between 1 and 6 hour.

These data indicate that both 2.5% and 10% serum induce *c-myc* expression in HPMC. In presence of 2.5% serum, however, *c-myc* seems to be induced more rapidly than in the presence of 10% serum.

2. Effects of Growth factors:

2a. Effects of EGF, TGFβ_1, and their combination on expression of *c-fos* mRNA

The autoradiograms showing the expression of *c-fos* in HPMC following treatment with media containing EGF, TGFβ_1, or their combination (EGF+TGFβ_1) are depicted in figure 17.

When HPMC were treated with EGF alone or in combination with TGFβ_1, the signal for *c-fos* mRNA was expressed between 30 minutes and 1 hour, but disappeared thereafter. In contrast, when exposed to TGFβ_1 alone cells did not show signal for *c-fos* expression.

The densitometry evaluation on *c-fos* expression revealed that following treatment of HPMC with EGF, the signal was reduced by 68% between 30 minutes and 1 hour. In contrast, when HPMC were co-treated with EGF and TGFβ_1 the expression of *c-fos* mRNA increased 44% between 30 minutes and 1 hour.

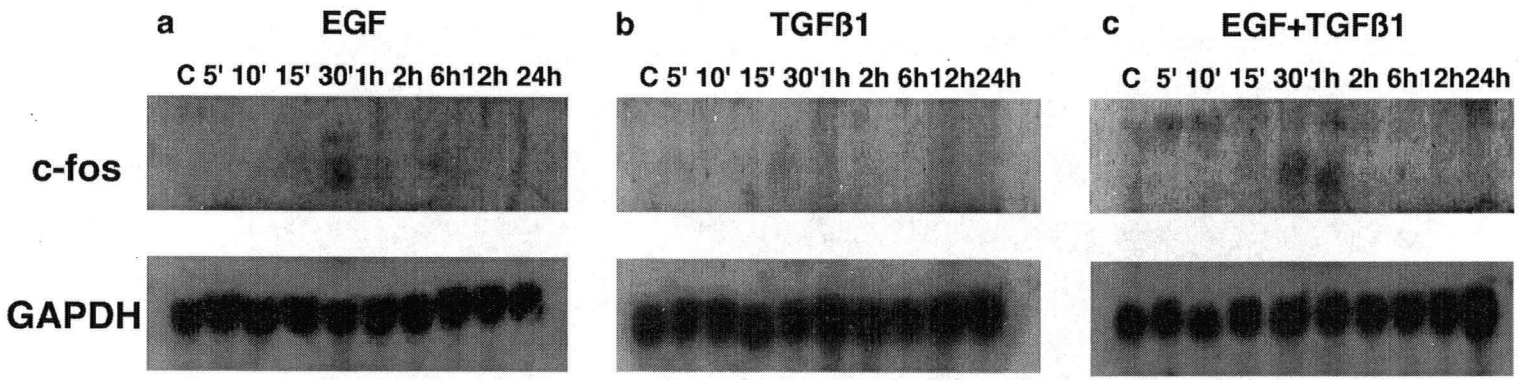


Figure 17. Northern blot analysis of effects of EGF(a), TGFβ1(b), or EGF+TGFβ1(c) on expression of *c-fos* mRNA in HPMC. HPMC were serum-starved for 24 hours, and then treated with DMEM containing growth factor(s) for 5, 10, 15, or 30 minutes, or 1, 2, 6, 12, or 24 hours. The controls were treated with DMEM alone. To perform Northern analysis, RNA was extracted from HPMC. An aliquot of 15 μg RNA was loaded per lane, separated on 1.2% agarose gel, transferred onto a nylon membrane, which was then hybridized using ^{32}P-labelled probes. *c-fos* expression was detected at 2.2kb. GAPDH gene expression (1.8kb) was used as a control.

The effects of EGF and TGFβ_1 on *c-fos* expression were further assessed in the presence of 2.5% serum. The autoradiograms showing the expression of *c-fos* in HPMC following treatment with media containing EGF+2.5% serum, TGFβ_1+2.5% serum, or their combination (EGF+TGFβ_1)+2.5% serum are depicted in figure 18.

In the presence of EGF+2.5% serum, or TGFβ_1+2.5% serum, HPMC showed signal for *c-fos* mRNA expression only at 1 hour. On the other hand, when HPMC were co-treated with EGF and TGFβ_1 in the presence of 2.5% serum, *c-fos* mRNA was expressed at 30 minutes. The intensity of *c-fos* expression then increased by 1 hour, and subsequently reduced at 2 hours before disappearing.

The densitometry data showed that between 30 minutes and 1 hour post-treatment, the increase in the intensity of *c-fos* expression in EGF+TGFβ_1+2.5% serum treated HPMC was 3.2 fold.

These results indicate that EGF alone or in combination with serum and/or TGFβ_1 induces *c-fos* transcription in HPMC, within 30 minutes to 1 hour. In contrast, TGFβ_1 alone does not support *c-fos* expression in these cells.

2b. Effects of EGF, TGFβ_1, and their combination on expression of *c-jun* mRNA

The autoradiograms showing the expression of *c-jun* in HPMC following treatment with media containing EGF, TGFβ_1, or their combination (EGF+TGFβ_1) are presented in figure 19.

Following treatment of HPMC with EGF alone, *c-jun* was expressed between 30 (very faintly) minutes and 1 hour, but subsequently disappeared. When treated with TGFβ_1 alone, HPMC did not show expression of *c-jun* mRNA. On the other hand, when HPMC were exposed to a combination of EGF and TGFβ_1, *c-jun* was expressed within 30 minutes, but at

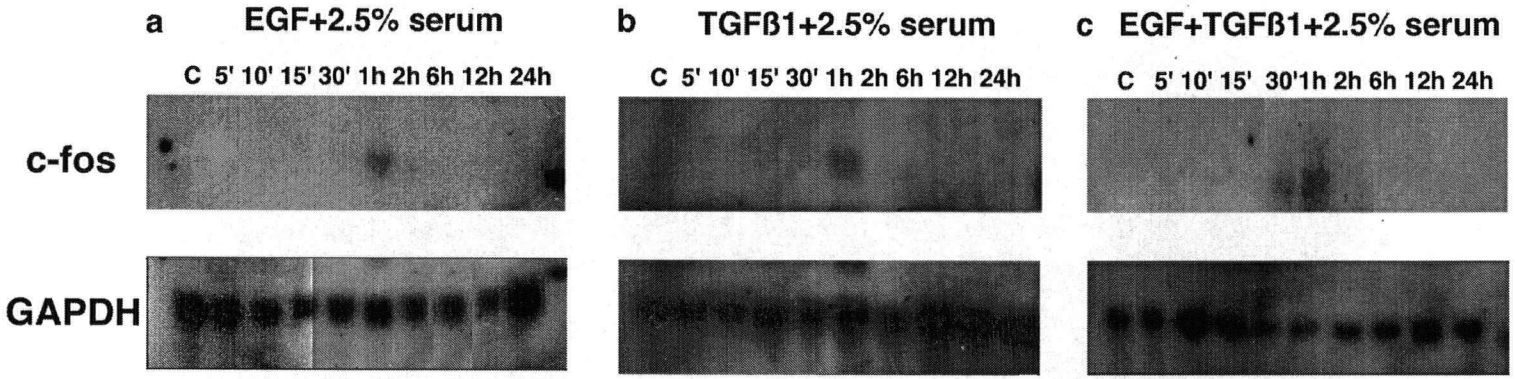


Figure 18. Northern blot analysis of effects of EGF+2.5% serum(a) , TGFβ1+2.5% serum(b), and EGF+TGFβ1+2.5% serum(c) on expression of *c-fos* mRNA in HPMC. HPMC were serum-starved for 24 hours, and then treated with DMEM containing growth factor(s) and 2.5% serum for 5, 10, 15, or 30 minutes, or 1, 2, 6, 12, or 24 hours. The controls were treated with DMEM alone. To perform Northern analysis, RNA was extracted from HPMC. An aliquot of 15 μg RNA was loaded per lane, separated on 1.2% agarose gel, transferred onto a nylon membrane, which was then hybridized using ^{32}P-labelled probes. *c-fos* expression was detected at 2.2kb. GAPDH gene expression (1.8kb) was used as a control.

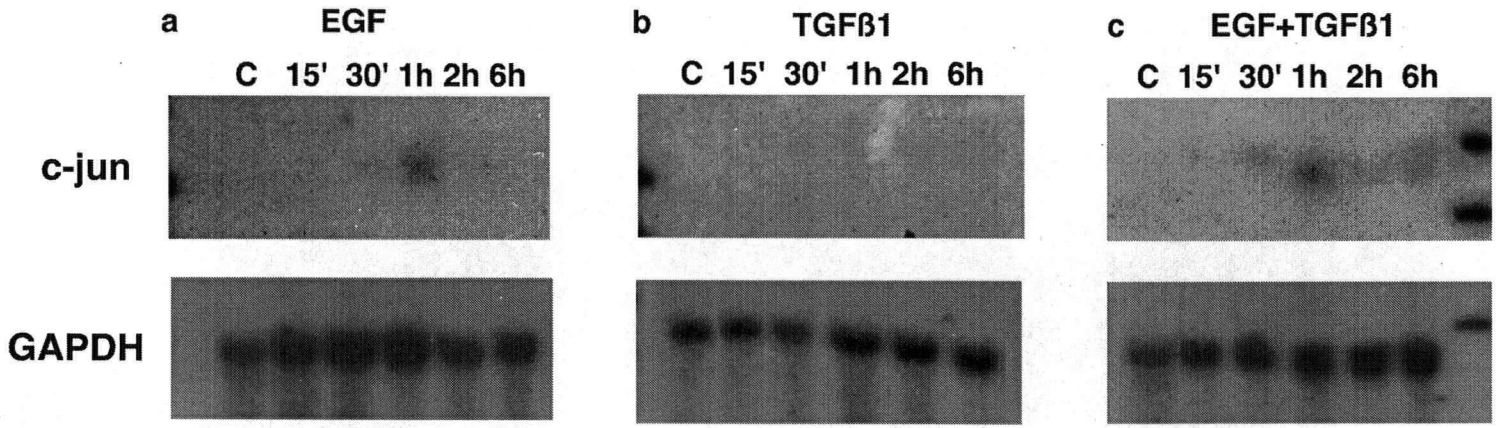


Figure 19. Northern blot analysis of effects of EGF(a), TGFβ1(b), or EGF+TGFβ1(c) on expression of *c-jun* mRNA in HPMC. HPMC were serum-starved for 24 hours, and then treated with DMEM containing growth factor(s) for 15, or 30 minutes, or 1, 2, or 6 hours. The controls were treated with DMEM alone. To perform Northern analysis, RNA was extracted from HPMC. An aliquot of 25 μg RNA was loaded per lane, separated on 1.2% agarose gel, transferred onto a nylon membrane, which was then hybridized using ^{32}P-labelled probes. *c-jun* expression was detected at 2.7kb. GAPDH gene expression (1.8kb) was used as a control.

very low levels. Subsequently, *c-jun* signal peaked at 1 hour, and was detected at least until 6 hours post-treatment, albeit with low intensity.

The densitometry analysis showed that when HPMC were treated with EGF alone, the intensity of *c-jun* signal increased 3.2 fold between 30 minutes and 1 hour post-treatment. In contrast, when cells were treated with both EGF and $TGF\beta_1$, the intensity of *c-jun* expression increased 2.4 fold between 30 minutes and 1 hour, and then decreased by 72% at 6 hours post-treatment.

The effects of growth factors on *c-jun* mRNA expression in HPMC were also examined in the presence of 2.5% serum. The autoradiograms showing the expression of *c-jun* in HPMC following treatment with media containing EGF+2.5% serum, $TGF\beta_1$+2.5% serum, or combination of EGF+$TGF\beta_1$+2.5% serum are shown in figure 20.

Following treatment of HPMC with EGF+2.5% serum, *c-jun* expression was observed between 1 and 2 hours post-treatment (figure 20a). Exposure of HPMC with $TGF\beta_1$+2.5% serum, however, did not show any *c-jun* expression (figure 20b). On the other hand, cells co-treated with EGF, $TGF\beta_1$ and 2.5% serum expressed *c-jun* mRNA within 15 minutes. Subsequently, the intensity of signal increased until 1 hour, and then gradually declined by 6 hours post treatment (figure 20c).

The densitometry analysis suggested that EGF+2.5% serum-exposed HPMC showed 52% reduction in *c-jun* mRNA expression between 1 and 2 hour post-treatment. On the other hand, following simultaneous exposure of cells to EGF, $TGF\beta_1$, and 2.5% serum the intensity of *c-jun* mRNA signal increased 3.75 fold between 15 minutes and 1 hour, and then declined by 30% at 6 hours post-treatment.

These data suggest that EGF alone, or in association with $TGF\beta_1$ and/or 2.5% serum triggers *c-jun* transcription in HPMC. Furthermore, although the peak of *c-jun* expression always remains at 1 hour, when cells are treated with a combination of EGF and $TGF\beta_1$

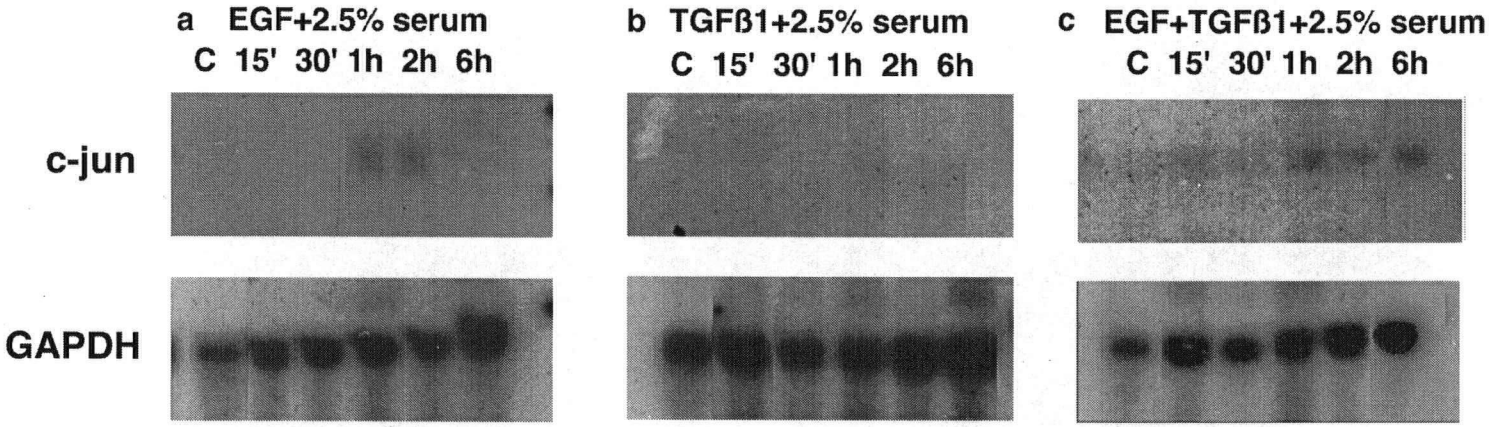


Figure 20. Northern blot analysis of effects of EGF+2.5% serum(a) , TGFβ1+2.5% serum(b), and EGF+TGFβ1+2.5% serum(c) on expression of *c-jun* mRNA in HPMC. HPMC were serum-starved for 24 hours, and then treated with DMEM containing growth factor(s) and 2.5% serum for 15, or 30 minutes, or 1, 2, or 6 hours. The controls were treated with DMEM alone. To perform Northern analysis, RNA was extracted from HPMC. An aliquot of 25 µg RNA was loaded per lane, separated on 1.2% agarose gel, transferred onto a nylon membrane, which was then hybridized using ^{32}P-labelled probes. *c-jun* expression was detected at 2.7kb. GAPDH gene expression (1.8kb) was used as a control.

and/or 2.5% serum, the *c-jun* mRNA signal tends to be sustained for long duration. In contrast, TGFβ_1 alone or in combination with 2.5% serum does not support *c-jun* expression in HPMC.

2c. Effects of EGF, TGFβ_1, and their combination on expression of *c-myc* mRNA

The autoradiograms showing the expression of *c-myc* in HPMC following treatment with media containing EGF, TGFβ_1, or their combination (EGF+TGFβ_1) are presented in figure 21.

Following treatment of HPMC with EGF, *c-myc* signal was observed at 30 minutes. The signal intensity then increased at 1 hour post-treatment, but disappeared altogether thereafter (figure 21a). After treatment of cells with TGFβ_1, however, expression of *c-myc* was not detectable (figure 21b). On the other hand, simultaneous treatment of HPMC with EGF and TGFβ_1 resulted in expression of *c-myc* mRNA between 1 and 6 hours post-treatment (figure 21c).

The densitometry data showed that following treatment of HPMC with EGF alone, there was a 3.6 fold increase in the intensity of *c-myc* signal between 30 minutes and 1 hour. In contrast, when cells were treated with EGF and TGFβ_1 simultaneously, *c-myc* expression declined by 37% between 1 hour and 6 hours post-treatment.

Furthermore, the effects of growth factors on *c-myc* mRNA expression in HPMC were examined in the presence of 2.5% serum. The autoradiograms showing the expression of *c-myc* in HPMC following treatment with media containing EGF+2.5% serum, TGFβ_1+2.5% serum, or combination of EGF+TGFβ_1+2.5% serum are presented in figure 22.

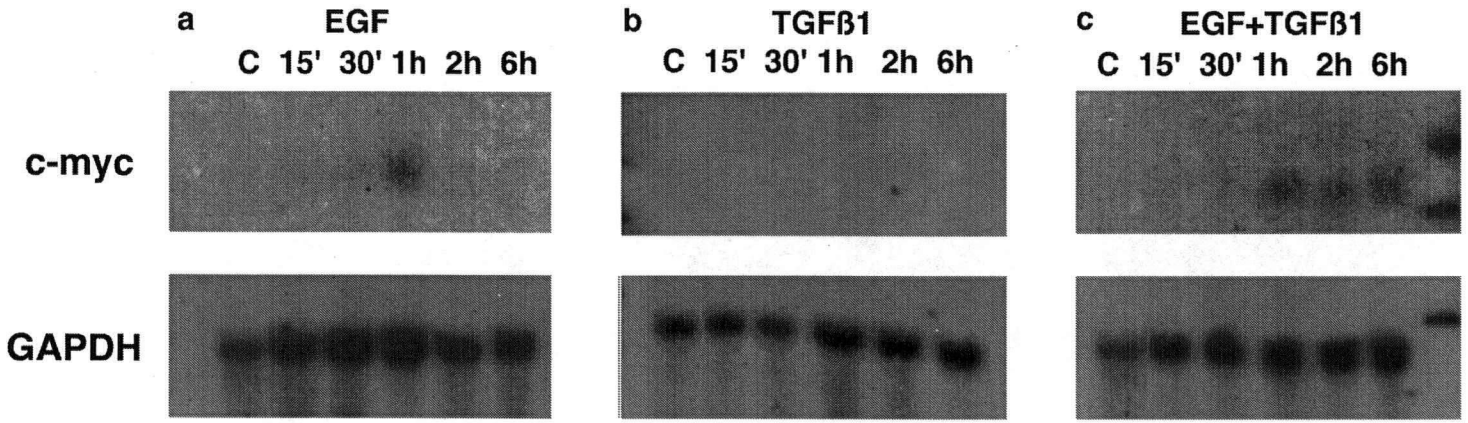


Figure 21. Northern blot analysis of effects of EGF(a), TGFβ1(b), or EGF+TGFβ1(c) on expression of *c-myc* mRNA in HPMC. HPMC were serum-starved for 24 hours, and then treated with DMEM containing growth factor(s) for 15, or 30 minutes, or 1, 2, or 6 hours. The controls were treated with DMEM alone. To perform Northern analysis, RNA was extracted from HPMC. An aliquot of 25 μg RNA was loaded per lane, separated on 1.2% agarose gel, transferred onto a nylon membrane, which was then hybridized using ^{32}P-labelled probes. *c-myc* expression was detected at 2.3kb. GAPDH gene expression (1.8kb) was used as a control.

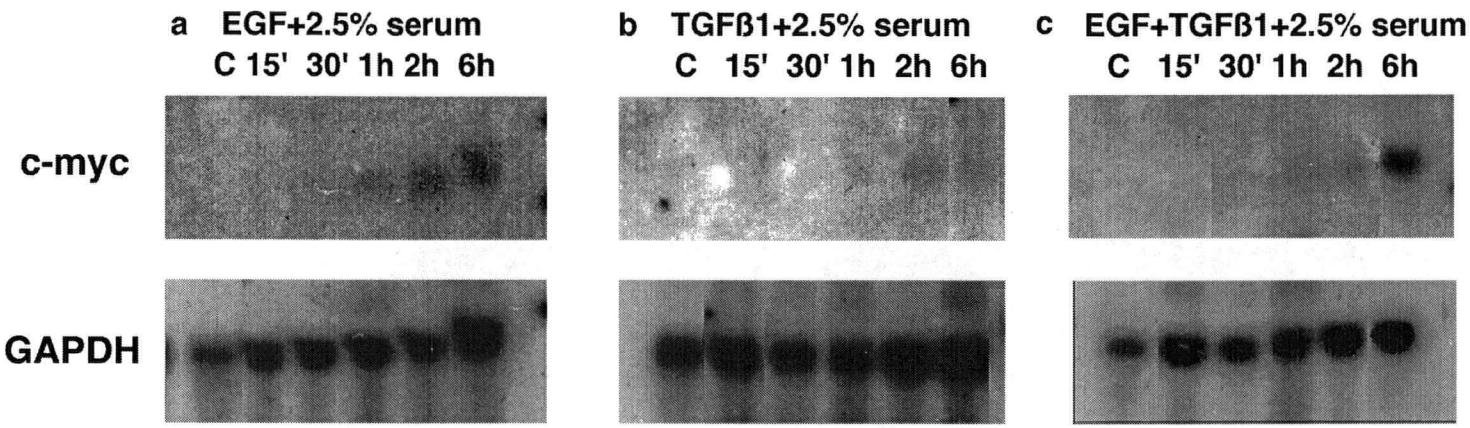


Figure 22. Northern blot analysis of effects of EGF+2.5% serum(a) , TGFβ1+2.5% serum(b), and EGF+TGFβ1+2.5% serum(c) on expression of *c-myc* mRNA in HPMC. HPMC were serum-starved for 24 hours, and then treated with DMEM containing growth factor(s) and 2.5% serum for 15, or 30 minutes, or 1, 2, or 6 hours. The controls were treated with DMEM alone. To perform Northern analysis, RNA was extracted from HPMC. An aliquot of 25 μg RNA was loaded per lane, separated on 1.2% agarose gel, transferred onto a nylon membrane, which was then hybridized using ^{32}P-labelled probes. *c-myc* expression was detected at 2.3kb. GAPDH gene expression (1.8kb) was used as a control.

HPMC treated with either EGF+2.5% serum or TGFβ_1+2.5% serum showed *c-myc* mRNA expression between 1 and 6 hours post-treatment. The highest *c-myc* expression signal was at 2 hours post-treatment. Co-treatment of cells with EGF and TGFβ_1 in the presence of 2.5% serum also showed *c-myc* mRNA signal at 1 hour, which gradually increased and reached the highest level of intensity at 6 hours.

The densitometry data revealed that the intensity of *c-myc* expression in HPMC treated with EGF+2.5% serum increased 2.4 fold between 1 and 2 hours, and then declined by 35% at 6 hour post-treatment. Similarly, when cells were exposed to TGFβ_1+2.5% serum, the *c-myc* expression initially increased 2.3 folds between 1 and 2 hour, and reduced by 38% at 6 hours post-treatment. On the other hand, when cells were treated simultaneously with EGF, TGFβ_1 and 2.5% serum, the expression of *c-myc* mRNA increased gradually by 74% between 1 and 6 hours post-treatment.

These observations show that, whereas EGF alone or EGF+2.5% serum stimulates *c-myc* transcription, TGFβ_1 alone does not support *c-myc* expression in HPMC. However, when cells are treated with TGFβ_1 in the presence of EGF and/or 2.5% serum, *c-myc* expression is observed. Additionally, when cells are exposed with growth factors in the presence of 2.5% serum, *c-myc* expression is present for a longer time, in comparison to cells treated with EGF alone.

3. Effects of TGFβ_1 pre-treatment:

3a. Effects of TGFβ_1 pre-treatment on expression of *c-fos* mRNA

The autoradiograms depicting the expression of *c-fos* in HPMC following pre-treatment with TGFβ_1 for 30 minutes, followed by exposure to 2.5% serum, or EGF alone, or EGF+2.5% are shown in figure 23.

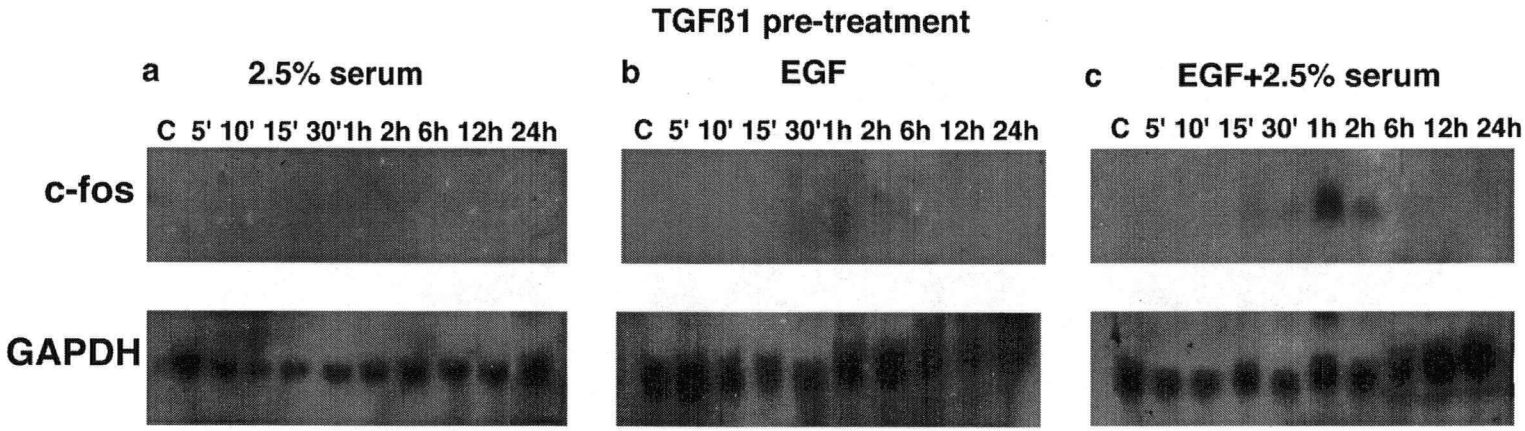


Figure 23. Northern blot analysis of effects of 2.5% serum(a) , EGF(b), and EGF+2.5% serum(c) on expression of *c-fos* mRNA in TGFβ1 pre-treated HPMC. HPMC were serum-starved for 24 hours, treated with TGFβ1 for 30 minutes, and then treated with DMEM containing 2.5% serum and/or EGF for 5, 10, 15, or 30 minutes, or 1, 2, 6, 12, or 24 hours. The controls (TGFβ1 exposed) were treated with DMEM alone. To perform Northern analysis, RNA was extracted from HPMC. An aliquot of 15 μg RNA was loaded per lane, separated on 1.2% agarose gel, transferred onto a nylon membrane, which was then hybridized using ^{32}P-labelled probes. *c-fos* expression was detected at 2.2kb. GAPDH gene expression (1.8kb) was used as a control.

Following pre-treatment with TGFβ_1 for 30 minutes, HPMC did not express *c-fos* mRNA (lane C, figures 23a, b, and c). Also, when the TGFβ_1 pre-treated cells were exposed to 2.5% serum, *c-fos* expression was not detected. In contrast, after exposure to EGF for 30 minutes or 1 hour, HPMC showed *c-fos* mRNA signal, which subsequently disappeared. Furthermore, when treated with EGF and 2.5% serum simultaneously, TGFβ_1 pre-treated cells expressed *c-fos* within 15 minutes post-treatment. The expression of mRNA peaked at 1 hour, declined by 2 hours and disappeared there after.

The densitometry data showed that when TGFβ_1 pre-treated HPMC were exposed to EGF, the *c-fos* signal intensity increased 25% between 30 minutes and 1 hour. Treatment of cells with EGF+2.5% serum, showed a 3 fold increase in *c-fos* mRNA expression between 15 minutes and 1 hour, followed by a 67% decline at 2 hours.

These results show that following pre-treatment of HPMC with TGFβ_1 for 30 minutes, exposure to 2.5% serum is not sufficient to trigger *c-fos* transcription. In contrast, when TGFβ_1 pre-treated cells are exposed to EGF alone or to EGF+2.5% serum, they express *c-fos* mRNA. In the presence of both EGF and 2.5% serum HPMC show more rapid and prolonged *c-fos* expression. Furthermore, in comparison to EGF treatment alone, the intensity of *c-fos* expression seems to be augmented in presence of both EGF+2.5% serum.

3b. Effects of TGFβ_1 pre-treatment on expression of *c-jun* mRNA

The autoradiograms showing the expression of *c-jun* in HPMC following pre-treatment with TGFβ_1 for 30 minutes, followed by exposure to 2.5% serum, EGF, and EGF+2.5% are depicted in figure 24.

When TGFβ_1-pre-treated HPMC were exposed to 2.5% serum, they expressed *c-jun* within 30 minutes. The expression of *c-jun* mRNA then increased at 1 hour, and then

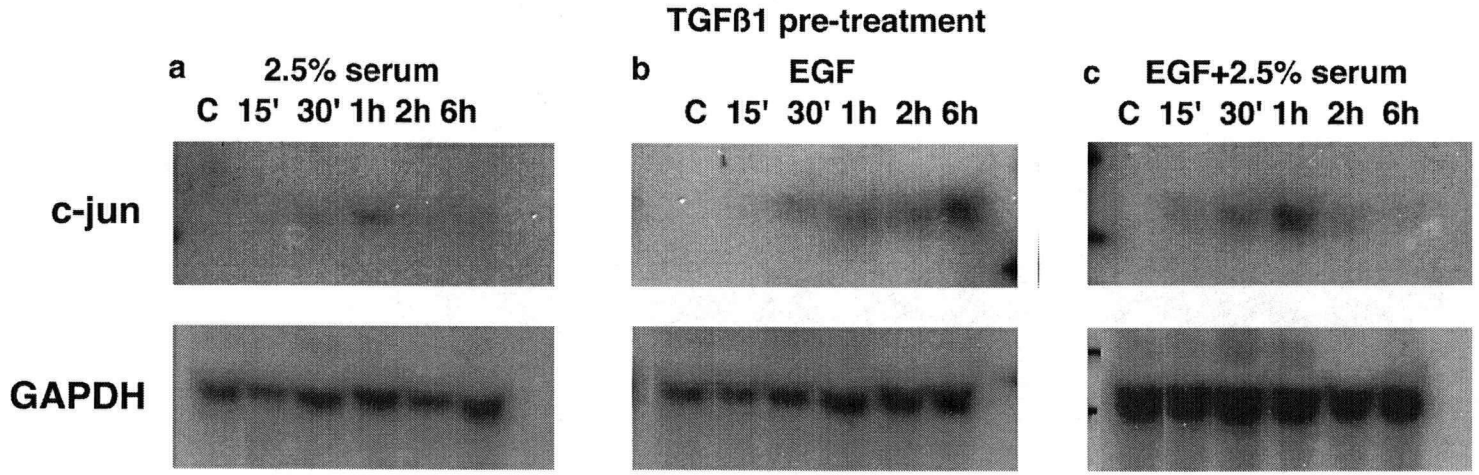


Figure 24. Northern blot analysis of effects of 2.5% serum(a) , EGF(b), and EGF+2.5% serum(c) on expression of *c-jun* mRNA in TGFβ1 pre-treated HPMC. HPMC were serum-starved for 24 hours, treated with TGFβ1 for 30 minutes, and then treated with DMEM containing 2.5% serum and/or EGF for 15, or 30 minutes, or 1, 2, or 6 hours. The controls (TGFβ1 exposed) were treated with DMEM alone. To perform Northern analysis, RNA was extracted from HPMC. An aliquot of 25 μg RNA was loaded per lane, separated on 1.2% agarose gel, transferred onto a nylon membrane, which was then hybridized using ^{32}P-labelled probes. *c-jun* expression was detected at 2.7kb. GAPDH gene expression (1.8kb) was used as a control.

gradually declined by 6 hour post-treatment. Treatment of cells with EGF alone also resulted in expression of *c-jun* between 30 minutes and 6 hours; however, *c-jun* expression intensity was highest at 6 hours post-treatment. On the other hand, exposure of TGFβ_1 pre-treated cells to EGF and 2.5% serum simultaneously showed *c-jun* transcription between 15 minutes and 6 hours post-treatment, with a peak at 1 hour.

The results of densitometry analysis revealed that when TGFβ_1 pre-treated HPMC were exposed to 2.5% serum, *c-jun* expression increased 50% between 30 minutes and 1 hour. Subsequently, the gene expression was gradually decreased by 58% at 6 hours post-treatment. When TGFβ_1 pre-treated cells were treated with EGF, *c-jun* signal intensity increased 42% between 30 minutes and 1 hour, and then a further 2 fold between 2 and 6 hours post-treatment. On the other hand, when treated with EGF+2.5% serum, *c-jun* expression increased 3.5 fold between 15 minutes and 1 hour, and subsequently decreased 87% at 6 hours after treatment.

These results indicate that exposure of HPMC to EGF, 2.5% serum, or EGF+2.5% serum, following TGFβ_1 pre-treatment, stimulates *c-jun* transcription in these cells. When HPMC are treated with both EGF and 2.5% serum *c-jun* expression at 1 hour is further augmented, in comparison to the condition when TGFβ_1 pre-treated HPMC are treated with EGF or 2.5% serum alone.

3c. Effects of TGFβ_1 pre-treatment on expression of *c-myc* mRNA

The autoradiograms showing the expression of *c-myc* in HPMC following pre-treatment with TGFβ_1 for 30 minutes, followed by exposure to 2.5% serum, EGF, and EGF+2.5% are presented in figure 25.

When TGFβ_1 pre-treated HPMC were exposed to 2.5% serum, they expressed *c-myc* mRNA between 1 to 6 hours post-treatment. However, when pre-treated cells were treated

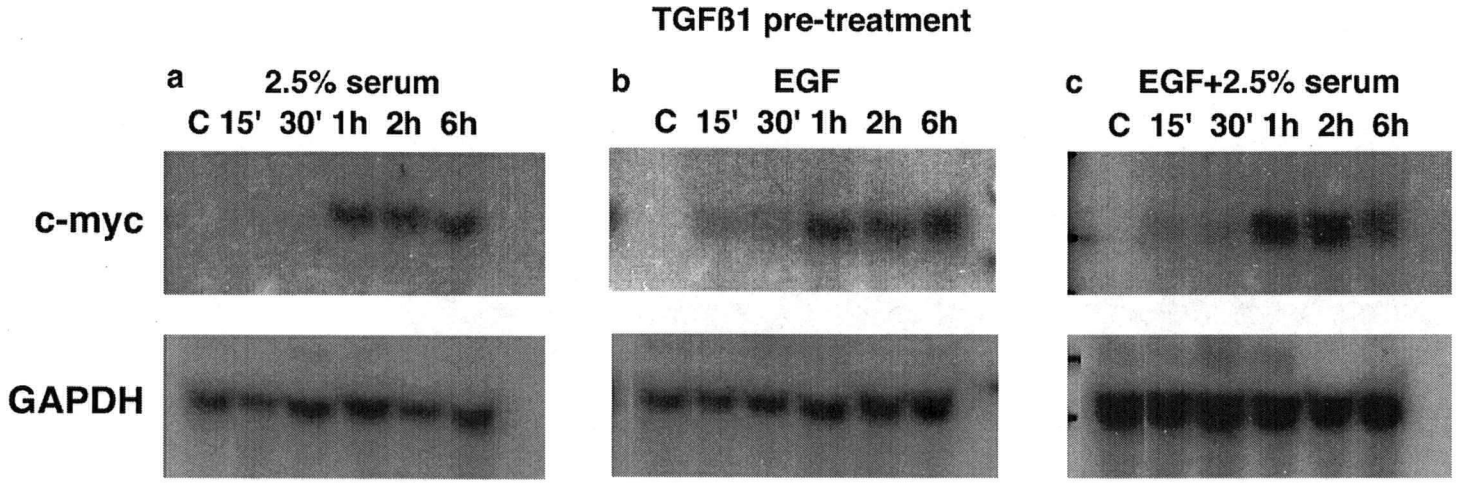


Figure 25. Northern blot analysis of effects of 2.5% serum(a) , EGF(b), and EGF+2.5% serum(c) on expression of *c-myc* mRNA in TGFβ1 pre-treated HPMC. HPMC were serum-starved for 24 hours, treated with TGFβ1 for 30 minutes, and then treated with DMEM containing 2.5% serum and/or EGF for 15, or 30 minutes, or 1, 2, or 6 hours. The controls (TGFβ1 exposed) were treated with DMEM alone. To perform Northern analysis, RNA was extracted from HPMC. An aliquot of 25 μg RNA was loaded per lane, separated on 1.2% agarose gel, transferred onto a nylon membrane, which was then hybridized using ^{32}P-labelled probes. *c-myc* expression was detected at 2.3kb. GAPDH gene expression (1.8kb) was used as a control.

with EGF alone, or EGF+2.5% serum, *c-myc* expression was observed within 15 minutes. The intensity of *c-myc* signal in both groups increased by 1 hour and persisted at least up to 6 hours post-treatment.

The densitometry data showed that in 2.5% serum-treated HPMC, there was a 2.5 fold increase in the intensity of *c-myc* expression between 1 and 2 hours post-treatment, followed by a 75% decline by 6 hours. On the other hand, EGF-treated cells showed 3 fold increase in *c-myc* expression between 15 minutes and 1 hour. The signal subsequently decreased by 40% at 6 hours post-treatment. Following treatment of $TGF\beta_1$-exposed HPMC with EGF+2.5% serum, there was a 10 fold increase in *c-myc* signal intensity between 15 minutes and 1 hour, followed by an additional 1.5 fold increase between 1 and 2 hours post-treatment. The signal intensity subsequently declined by 53% at 1 hour.

These observations suggest that 2.5% serum, EGF, or their combination is sufficient to trigger *c-myc* expression in $TGF\beta_1$ pre-treated HPMC. In comparison to EGF or 2.5% serum alone, treatment of cells with a combination of EGF and 2.5% serum, seems to promote highest expression of *c-myc* within 2 hours.

Discussion

In order to analyze proliferative behavior of embryonic secondary palate mesenchymal cells, free from the complexities of the *in vivo* environment, a primary cell culture system was used in the present study. The results showed that HPMC were capable of survival and sustained growth when seeded on plastic culture plates. Previously, other investigators have made similar observations on embryonic palate mesenchymal cells from other mammals (human, mice, and rat) (Yoneda and Pratt, 1981; Wee et al., 1981, Greene et al., 1981b; Chepenik and Greene, 1981; Zimmerman et al., 1983; Kukita and Kurisu, 1986; Yano et al., 1996b), and bird (quail) (Izadnegahdar et al., 1995; Hehn et al., 1996). Indeed, recent studies have shown that palate mesenchymal cells proliferate more rapidly on a plastic surface than those on or within an ECM substratum (Sharpe et al., 1992; 1993; Dixon et al., 1993a, b). In addition, primary cultures of embryonic cells obtained from mouse and chick limb bud mesenchymal cells (Paulsen and Solursh, 1988; Biddulph and Dozier, 1989; Capehart and Biddulph, 1991), rat lung fibroblast (Nunez and Torday, 1995), skin mesenchyme (Polakiewicz et al., 1992), and chick mandibular, maxillary, and frontonasal mesenchyme (Langille et al., 1989) have been shown to grow well on plastic surfaces. The results of the present study further showed that the rate of proliferation of HPMC was dependent on the concentration of serum in the culture media: the rate of proliferation was highest in the presence of 10% serum and lowest in 2.5% serum. In the presence of 1% serum, HPMC did not grow. Previous studies on embryonic mouse palate mesenchymal cells also suggested that the presence of at least 2.5% serum in culture media was required for survival and sustained growth of cells (Sharpe et al., 1992a, b; 1993; Dixon et al., 1993a, b). These observations suggest that culture conditions are important determinants of *in vitro* growth behavior of embryonic palate mesenchymal cells.

Next, the effects of growth factors EGF, TGFβ_1, or their combination, on the proliferative behavior of HPMC, were evaluated by growth curve analysis and measurement of DNA synthesis (as determined by ^{3}H-thymidine incorporation). Several studies have previously reported the mitogenic effects of EGF on embryonic mammalian palate mesenchymal cells (Yoneda and Pratt, 1981; Kukita and Kurisu, 1986; Kukita et al., 1987; Pisano and Gereene, 1987; Chepenik and Gunwald, 1988; Gawel-Thompson and Greene, 1989; London et al., 1989; Sharpe et al., 1992a, b; Dixon et al., 1993b; Chepenik et al., 1994). The data of the present study, indicated that, when treated at sub-confluent stage, EGF accelerated the proliferation of HPMC, but did not enhance DNA synthesis in 24 hours. When HPMC were treated with EGF in the presence of 2.5% serum, however, DNA synthesis was increased compared to the controls. Also, the presence of serum seem to be essential for EGF to exert its mitogenic effects on sub-confluent (Sharpe et al., 1992b; Dixon et al., 1993b, present study), but not on confluent cultures of mammalian embryonic palate mesenchymal cells (Yoneda and Pratt, 1981; Kukita and Kurisu, 1986; Kukita et al., 1987; London et al., 1989). The current analysis of the effects of EGF on palate mesenchymal cells supports the previously held notion that in order to stimulate cell cycle under sub-confluent culture condition, EGF may require the presence of other factor(s) in serum (Stiles et al., 1979). Taken together, these data support the hypothesis that EGF is a positive regulator of HPMC proliferation.

In contrast to EGF, TGFβ_1 inhibited DNA synthesis and arrested proliferation of HPMC. These findings are in line with previously reported data in the literature, in which TGFβ_1, in the presence or absence of serum, was shown to inhibit proliferation of embryonic palate mesenchymal cells in mice (Linask et al., 1991; Sharpe et al., 1992a, b), as well as of embryonic rat and human fibroblasts, and rat intestinal epithelial cells in primary culture (Anzano et al., 1986; Booth et al., 1995; Kletsas et al., 1995). It is well

recognized in the literature that TGFβ_1 is perhaps one of the best known physiological inhibitors of cell proliferation (Moses and Leof, 1986; Roberts and Sporn, 1990; Massagué and Polyak, 1995). TGFβ_1 is able to inhibit both *in vivo* and *in vitro* growth of several different cell types including epithelial, endothelial, fibroblast, neuronal, lymphoid, and hematopoietic cells (Massagué, 1992). A number of studies, however, have also shown that TGFβ_1 exerts mitogenic effects on some cell types . For instance, TGFβ_1 has been shown to induce proliferation of human embryonic palate mesenchymal cells (perhaps due to the altered phenotype of these cells) (Linask et al., 1991), as well as of senescent human fibroblasts, corneal endothelial cells, or smooth muscle cells (Kletsas et al., 1995; Rieck et al., 1995). In addition, TGFβ_1 has been reported to accelerate proliferation of established fibroblast cell lines (such as NRK, AKR-2B, and Rat-1), as well as transformed cells (such as lung carcinoma) (Roberts et al., 1985, Moses and Leof, 1986). It has been suggested that the mitogenic effects of TGFβ_1 may occur indirectly possibly through induction of other mitogenic molecules such as PDGF, FGF, or their receptors (Leof et al., 1986; Plouët and Gospodarowicz, 1989; Kletsas et al., 1995; Reick et al., 1995). Thus, the positive or negative effects of TGFβ_1 on cell proliferation appears to be complex, and seems to depend on cell type, stage of phenotypic differentiation, and the availability of other factors in culture medium.

The data of the present study also demonstrated that when sub-confluent HPMC were treated with both EGF and TGFβ_1 simultaneously, TGFβ_1 overcame the mitogenic effects of EGF. These observations are in line with the data reported by Sharpe and colleagues (1992a) in murine palate mesenchymal cells, and suggest that interaction among growth factors may be important in regulating the proliferation of palate mesenchymal cells. Previously, TGFβ_1 has been shown to antagonize the mitogenic effects of serum or exogenous growth factors such as EGF, PDGF, or FGF in cultured fibroblasts, endothelial, epithelial,

and neuronal cells (Takehara et al, 1987; Coffey et al., 1988; Mulder et al., 1990; Yoshiura et al., 1994; Kletsas et al., 1995; Vergelli et al., 1995). In general, however, the inhibitory effect of TGFβ_1 on cell proliferation of most cell types seems to be reversible, and cells resume growth upon removal of TGFβ_1 from culture media (Moses and Leof., 1986; Polyak, 1996).

To further study whether the anti-proliferative effect of TGFβ_1 on HPMC was reversible or not, the cells were pre-treated with TGFβ_1. The results showed that the anti-proliferative effect of TGFβ_1 was exerted rapidly: pre-treatment of HPMC with TGFβ_1 for 30 minutes was sufficient to inhibit both serum- and/or EGF-induced DNA synthesis and proliferation. Also, these data indicated that the effects of TGFβ_1 on the proliferative behavior HPMC was irreversible. After 30 minutes of exposure to TGFβ_1, and its subsequent removal from the culture media, the growth of HPMC was arrested and they were unable to respond to the mitogenic effects of serum and/or EGF. Pre-treatment of HPMC with TGFβ_1 for different durations of up to 12 hours also resulted in consistent repression of DNA synthesis. Previously, Sharpe and colleagues (1992a) also observed inhibition of DNA synthesis following TGFβ_1 pre-treatment of murine embryonic palate mesenchymal cells for 24 hours. An irreversible inhibitory effect of TGFβ_1 on the proliferation of endothelial cells (Takehara et al., 1987) and myoblasts (Zentella and Massagué, 1992) have also been noted.

The foregoing analyses suggest that the presence of at least 2.5% serum is required for survival and sustained growth of HPMC in primary culture. In addition, whereas EGF is a positive regulator of DNA synthesis and proliferation of these cells, TGFβ_1 arrest the proliferation of HPMC. TGFβ_1 also prevents the proliferative response of HPMC to EGF and/or serum, suggesting that interaction among growth factors may play an important role in regulation of proliferation, and thus cell cycle progression, of HPMC.

It is now well recognized in the literature that the regulation of cell proliferation requires sequential activation of several interacting intracellular signaling pathways that subsequently induce series of immediate early genes putatively involved in regulation of various events determining cell cycle progression (Edwards, 1994; Seger and Krebs, 1995). The discussion so far has focused on the ability of EGF and TGFβ_1 to modulate the proliferation of embryonic palate mesenchymal cells. However, there are no reports in the literature analyzing their effects on the expression of immediate early genes in these cells. The present study provides the first report on expression of immediate early genes, and their modulation by growth factors, in embryonic palate mesenchymal cells.

The results of the Northern blot analysis indicated that under serum starved conditions, transcripts of *c-fos*, *c-jun*, and *c-myc* were undetectable in HPMC. These data are consistent with the observation that the mRNA levels of these proto-oncogenes are at extremely low levels in quiescent fibroblasts (Kelly et al., 1983; Coffey et al., 1988; Waters et al., 1991; Campisi, 1992; Kim et al., 1993), indicating that serum starved cells were in G0 state.

Treatment of quiescent HPMC with serum resulted in rapid but transient induction of all three proto-oncogenes whose transcript size were comparable to those found in other cell types (Müller et al., 1984; Almendral et al., 1988). When HPMC were exposed to 2.5% or 10% serum, expression of *c-fos* mRNA occurred within 15-30 minutes, but the signal disappeared by 2 hours. On the other hand, *c-jun* expression was not detectable with 2.5% serum. In presence of 10% serum, however, *c-jun* mRNA was induced within 15 minutes, and subsequently declined at 6 hour, thus further reinforcing the proposition made earlier in the Discussion that culture conditions play an important role in regulation of specific gene expression in embryonic palate mesenchymal cells. Treatment of HPMC with 2.5% or 10% serum also induced *c-myc* expression by 30-60 minutes, which peaked at 1

hour, and gradually declined at 6 hours. These observations on the induction of immediate early genes in HPMC by serum corroborate those in quiescent fibroblasts where similar rapid and transient induction of immediate early genes were associated with cell proliferation (Greenberg and Ziff, 1984; Lau and Nathans, 1985; 1987; Almendral et al., 1988; Ryseck et al., 1988). Since, in the present study, serum treatment also induces both the immediate early genes and proliferation in HPMC in that order, it is plausible that induction of these genes may be required for the transition of HPMC from a quiescent to proliferating state.

Previously, mitogens such as EGF, PDGF, and FGF have been shown to induce expression of *c-fos*, *c-jun*, and *c-myc* quiescent fibroblasts as efficiently as serum (Kelly et al., 1983; Cochran et al., 1984; Kruijer et al., 1984; Müller et al., 1984; Quantin and Breathnatch; 1988; Ryseck et al., 1988; Hudson and Gill). The data of the present study also showed that treatment of serum-starved HPMC with EGF, both in presence and absence of 2.5% serum, induced expression of *c-fos*, *c-jun*, and *c-myc*. When cells were exposed to EGF alone, the expression all three proto-oncogenes were expressed rapidly and transiently. Similar observations have been made by Müller and associates (1984), in NIH 3T3 fibroblasts where *c-myc* mRNA reached basal levels rapidly after treatment with EGF alone. Expression of *c-jun* and *c-myc*, however altered depending on the presence or absence of serum in culture media: in the absence of serum, EGF was able to induce *c-jun* and *c-myc* signals only between 30 minutes and 1 hour, in the presence of 2.5% serum, EGF-induced expression of these genes was seen between 1 and 6 hours. Clearly, the presence or absence of serum appears to regulate the temporal expression of at least *c-myc* and *c-jun*, which, in turn, may be associated with the non-mitogenic response of serum-starved HPMC following their exposure to EGF alone. This would further support the notion expressed above that

simultaneous availability of other factors in culture media is essential for EGF-induced stimulation of proliferation of HPMC.

On the other hand, the results of the present study showed that in the absence of serum, TGFβ_1 was unable to induce immediate early gene expression in HPMC. Even though there are no reports in the literature on the effects of TGFβ_1 on expression of immediate early genes in embryonic mesenchymal cells in primary culture, previous studies on other cell types have shown that TGFβ_1 exerts diverse effects on expression of *c-fos, c-jun,* and *c-myc*. Whereas in some cell types, including BALB/MK keratinocytes, BALB 3T3 fibroblasts, Swiss 3T3 fibroblasts, and Pig leydig cells, TGFβ_1 alone does not affect proto-oncogene expression (Coffey et al., 1988; Hall et al., 1991; Chatani et al., 1995), in other cell types such as embryonic rat L2, NIH 3T3, ARK 2B, mouse embryonic fibroblasts, mink lung epithelial cells, and mouse keratinocytes, TGFβ_1 can rapidly stimulate or inhibit the expression these genes (Liboi et al., 1988; Petrovaraara et al., 1989; Pietenpol et al., 1990; Hall et al., 1991; Kim et al., 1993). In addition, the effect of TGFβ_1 on immediate early gene expression in different cell type does not correlate with its effects on cell proliferation. For example, *c-jun* expression was observed in both human adenocarcinoma cells, in which proliferation was inhibited by TGFβ_1 and in AKR-2B mouse embryo fibroblasts, which were stimulated by TGFβ_1 (Petrovaara et al., 1989). Furthermore, TGFβ_1 stimulated the proliferation of BALB 3T3 and Swiss 3T3 fibroblasts without inducing *c-fos* expression (Chatani et al., 199). wheras in endothelial cells TGFβ_1 induced *c-fos* expression, but inhibited cell proliferation (Takehara et al.., 1987). Thus, the effect of TGFβ_1 on immediate early gene expression, like that on cell proliferation, also seem to be varied depending on cell types and culture conditions.

To further analyse whether the TGFβ_1 arrest of serum- or EGF-induced HPMC proliferation had affected the expression of the proto-oncogenes, the effects of growth factor

combination and TGFβ_1 pre-treatment on *c-fos*, *c-jun*, and *c-myc* expression were examined. When cells were treated with TGFβ_1 in the presence of 2.5% serum, mRNA expression was observed for *c-fos* (peak at 1 hour) and *c-myc* (peak at 2 hour), but not *c-jun,* indicating that TGFβ_1 does not interfere with serum-induced expression of immediate early genes.

Following co-treatment of HPMC with EGF and TGFβ_1 in the presence or absence of 2.5% serum, the mRNA of all three genes was expressed, indicating that abrogation of mitogenic response of EGF by TGFβ_1 may have not been exerted through inhibition of immediate early gene expression. Earlier literature also demonstrates that the growth inhibitory effect of TGFβ_1 on mitogen-induced proliferation of different cell types does not inhibit mitogen-induced *c-fos* or *c-jun* expression. For example, TGFβ_1 inhibits mitogen-induced proliferation of hamster lung fibroblasts (Chambard and Puyssegur, 1988), neonatal human fibroblasts (Paulsson et al., 1988; Keltsas et al., 1995), BALB/MK keratinocytes (Coffey et al., 1988), rabbit gastric epithelial cells (Yoshiura et al., 1994), and human renal mesengial cells (Schoeckleman et al., 1997) in culture, without inhibiting mitogen-induced expression of *c-fos* or *c-jun* in these cells. Therefore, it is possible that in these cell types, including HPMC (present study), TGFβ_1 may arrest EGF-induced proliferation through other mechanisms, which do not interfere with mitogen-induced *c-fos* or *c-jun* expression.

In comparison to the effects of TGFβ_1 on mitogen-induced expression of *c-fos* and *c-jun*, the effect of this growth factor on *c-myc* expression seems to be dependent on the cell type. For instance, in HPMC (present study), hamster lung fibroblasts (Chambard and Pouyseggur, 1988), and rat intestinal epithelial cells (Ko et al., 1994), TGFβ_1 does not affect the mitogen-induced expression of *c-myc*, but in other cell types such as human mammary carcinoma (Franandez-Pol et al., 1987), BALB/MK keratinocytes, secondary

cultures of human keratinocytes (Coffey et al., 1988, Pientepol et al., 1990a, b; Munger et al., 1992), colon carcinoma cells (Mulder et al., 1990), or rabbit gastric epithelial cells (Yoshiura et al., 1994) TGFβ_1 reduced mitogen-induced *c-myc* expression. Also, over-expression of *c-myc* in keratinocytes leads to TGFβ_1 resistance (Alexandrov et al., 1995), further indicating that TGFβ_1 may be exerting its growth inhibitory effects through down-regulation of *c-myc* expression (Pientepol et al., 1990; Zentella et al., 1991; Munger et al., 1992), a proposition that is in contrast to the observations of the present study.

The data of the present study also revealed that co-treatment of HPMC with EGF and TGFβ_1 (in presence or absence of serum) affected the temporal expression of all three proto-oncogene mRNAs. For example, in contrast to exposure to EGF alone or EGF + serum, when *c-jun* was no longer detectable after 1-2 hours, co-treatment of HPMC with EGF and TGFβ_1 (± serum) resulted in expression of *c-jun* mRNA for up to 6 hours. Similarly, when HPMC were co-treated with EGF, TGFβ_1, and serum, *c-fos* expression persisted up to 2 hours in comparison to 1 hour duration following EGF/serum treatment. The expression of *c-myc* in HPMC was also prolonged in presence of both EGF and TGFβ_1, compared with EGF alone. Previously, several investigators have also reported sustained expression of mitogen-induced *c-fos* in the presence of TGFβ_1 in other cell types (Liboi et al.,1988; Paulsson et al., 1988; Kletsas et al., 1994). Whether the prolonged expression of immediate early genes in HPMC in the presence of TGFβ_1 is due to an increased rate of gene expression or a decreased rate of mRNA degradation, remains to be clarified. However, since TGFβ_1 by itself does not stimulate transcription of immediate early genes in HPMC, it is possible that TGFβ_1 might exert post-transcriptional effects on immediate early gene mRNA (Coffey et al., 1988) to prolong the expression of these mRNA transcripts. This could imply that the altered temporal expression of mitogen-induced immediate early genes by TGFβ_1 may be associated with growth arrest.

With the exception of *c-fos*, which was not stimulated by 2.5% serum in TGFβ_1 pre-treated HPMC, pre-treatment of HPMC with TGFβ_1 followed by treatment with 2.5% serum, EGF, or their combination, supported mRNA expression of all three proto-oncogenes, as appropriate. In addition, the mRNA expression of all three proto-oncogenes, although occurring very rapidly (within 15 minutes), was prolonged, with *c-myc* and *c-jun* expression lasting for up to at least 6 hours; and *c-fos* expression until 2 hours. Studies on the modulatory effect of TGFβ_1 pre-treatment on other growth factors are scant. Earlier, Miki and associates (1994) showed that 30 minutes of TGFβ_1 pre-treatment potentiated neurotransmitter-induced *c-fos* expression in myocardial cells. In endothelial cells, TGFβ_1 pre-treatment does not alter EGF-induced *c-fos* expression, but reduces *c-myc* expression (Takehara et al., 1987), whereas in endometrial carcinoma cells, it reduces *c-fos* expression within 1 hour of TGFβ_1 pre-treatment (Bergman et al., 1997). These observations would indicate that the effect of TGFβ_1 pre-treatment on mitogen-induced expression of immediate early genes may be cell type-specific.

The foregoing analysis of the data of the present study, along with that from literature clearly indicates that cooperation between EGF and TGFβ_1 is crucial in the regulation of proliferation of embryonic palate mesenchymal cells. The question that logically follows is how do EGF and TGFβ_1 exert their respective biological effects to regulate proliferation of palate mesenchymal cells?

Previous studies have shown that both the second-messenger dependent pathways involving PKA and PKC (Pisano and Greene, 1986; Pisano et al., 1986; Chepenik and Grunwald, 1988; Chepenik and Haystead, 1989), and second-messenger independent pathways involving CK2, MAPK, and p34cdc2 (Young et al., 1995; 1996a, b), allow transduction of extracellular messages from the cell surface to the nucleus to regulate cell proliferation and differentiation. These pathways are activated during normal development

of secondary palate in mammals as well as other vertebrates (Hehn et al., 1995; 1996 1997a, b). Although it remains to be determined during palate development, it has been shown that in nuclei of other eukaryotic cells, the signaling molecules stimulated by these pathways activate transcription factors, which, in turn, stimulate expression of immediate early genes (*c-fos*, *c-jun*, *c-myc*, etc.) that seem to be required for progression of cell into G1 phase, and for eventual regulation of cell proliferation (Triesman, 1994, 1996).

It has been shown that ligand-induced phosphorylations of protein kinases regulate the activation of early genes (Hunter and Karin, 1992). The pathways through which serum or growth factors such as EGF activate immediate early genes appear to be specific (Gupta, et al., 1996). For example, *c-fos* expression seems to be regulated through Ras-MAPK pathway (Janknecht et al., 1995), whereas tyrosine kinase *Src* is an upstream activator of only *c-myc* (Barone and Courtneidge, 1995). Exogenous EGF has been shown to bind to receptors on palate mesenchymal cells (Abbott et al., 1988; Shiota et al., 1990; Sharpe et al., 1992a), and stimulates their proliferation (Gawel-Thompson and Greene, 1989; Sharpe et al., 1992a, b; Shah et al., 1995a). EGF also activates PKC (Chepenik and Grunwald, 1988; Chepenik and Haystead, 1989), MAPK, and CK2 (Shah et al., 1995a, b; Young et al., 1996) in mammalian palate mesenchymal cells (figure 26). Thus, it is plausible that these EGF-induced signaling molecules may activate *c-fos*, *c-jun*, and *c-myc* expression (present study) and advance the palate mesenchymal cells in G1 and thus contribute to the stimulation of cell proliferation.

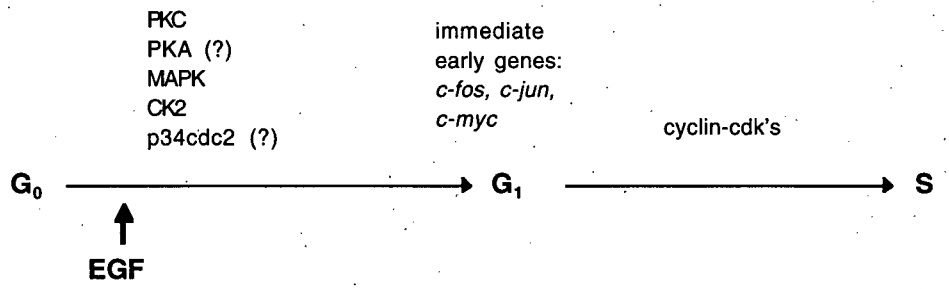


Figure 26. Induction of signaling cascades and immediate early genes as the palate mesenchymal cells advance from G0 through G1 phase of the cell cycle.

On the other hand, when TGFβ1 binds to embryonic palate mesenchymal cells (Linask et al., 1991), it suppresses their proliferation (Linask et al., 1991; Sharpe et al., 1992a, b; present study) and does not activate *c-fos*, *c-jun*, or *c-myc* expression in HPMC (figure 27; present study). Earlier, it was shown that TGFβ1 suppresses activation of both MAPK and CK2 in HPMC (Young et al., 1996). Taken together, these observations would suggest that lack of activation of immediate early genes in HPMC may be related to suppression of at least MAPK or CK2 signaling pathways.

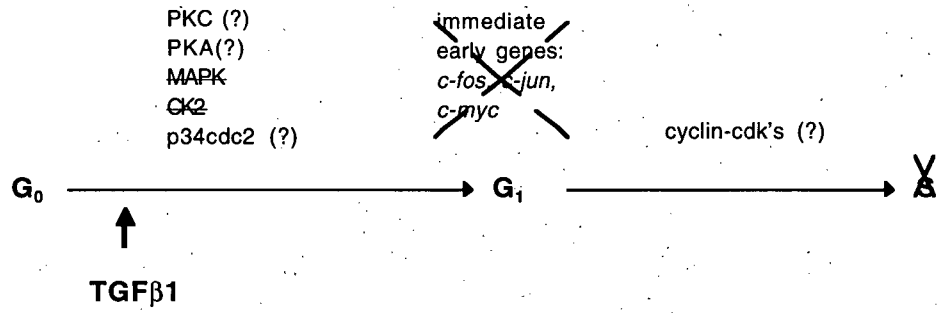


Figure 27. Effects of TGFβ1 on signaling molecules and immediate early genes as palate mesenchymal cells advance from G0 through G1 phase of the cell cycle.

In addition, although not yet verified during palate development, $TGF\beta_1$ has been implicated in suppression of activation of at least two other cell cycle controlling molecules,

p53 and retinoblastoma (Rb), as a part of the pathway that leads to the arrest of cell proliferation (Cox and Lane, 1995; Beijersbergen and Bernards, 1996; Milcezarek et al., 1997). Function of p53 is generally associated with the regulation of cell proliferation during tumor progression or cell injury (Deppert, 1994; Levine et al., 1991; Sager, 1992). Rb is a negative regulator of cell cycle; when hypophosphorylated, it binds to and inhibits transcription factor E2F, which is required for cell cycle progression (Sanchez and Dynlacht, 1996). Phosphorylation of Rb is mediated by protein complexes of G1 cyclins and cyclin dependent kinases (cdk) such as cyclin D/Cdk 4 or 6, and cyclin E/Cdk 2, whose sequential formation, activation and inactivation are necessary for cell cycle progression (Pardee, 1989; Sherr, 1993; 1994; 1995). $TGF\beta_1$ inhibits phosphorylation of Rb resulting in an accumulation of the under-phosphorylated functional form of the protein (Laiho et al., 1990; Munger, et al., 1992; Beijersbergen and Bernards, 1996). Also, $TGF\beta_1$ inhibition of Rb seem to be through suppression of the mRNA or protein levels of several G1 cyclins and Cdk's, or prevention of the cyclin/cdk complex activity (Massagué and Polyak, 1995; Yingling et al., 1995; Polyak, 1996) by regulating cyclin dependent kinase inhibitors (CKI's) (Polyak et al., 1994; Hannon and Beach, 1994; Datto et al., 1995). CKI's are low molecular weight proteins that bind cyclin-cdk complexes and inhibit their activities (Hunter and Pines, 1994; Sherr, 1994). The known CKI family members so far include, p21 (WAF/ Cip 1), p27 (Kip 1), p16 (INK 4/ MTS1) and p15 (INK 4B/MTS2). $TGF\beta_1$ treatment of cells seems to stimulate p15 gene expression which results in an increase in its protein levels (Hannon and Beach, 1994). At high cellular concentration, p15 associates with cyclin D-cdk4 and cdk6, and inhibits their activity during G1(Hannon and Beach, 1994). Similarly, $TGF\beta_1$ increases p21 gene expression, which at excess protein levels binds to, and inhibits cyclin D and cdk 2 (Datto et al., 1995). On the other hand, $TGF\beta_1$ seems to increase the p27 protein levels through a post-

translational mechanism (Polyak et al., 1994; Slingerland et al., 1994), and prevent formation of catalytically active cyclin E/cdk2 in mink lung epithelial cells (Koff et al., 1993). Thus, there appears to be multiple interacting mechanisms between $TGF\beta_1$, and G1 cyclin-cdk activities indicating that $TGF\beta_1$ may play an important role in regulating at least cyclin-cdk cell cycle machinery to exert its anti-proliferative effects. Clearly, information on $TGF\beta_1$ regulation of cyclin-cdk activity, and of CKI, is essential to further understand the involvement of $TGF\beta_1$ in regulation of proliferation of HPMC.

Following co- or pre-treatment with $TGF\beta_1$, EGF and/or serum does induce expression of all three immediate early genes but does not reverse the $TGF\beta_1$ suppressed proliferation of HPMC (present study). Earlier it was shown that when HPMC were co-treated with EGF and $TGF\beta_1$, MAPK activation was not affected but CK2 activation was (figure 28) (Young et al., 1996). It is plausible that EGF-stimulated MAPK in a timely manner could induce *c-fos, c-jun,* and *c-myc* expression, which was apparently insufficient to carry HPMC through G1 phase of the cell cycle possibly because other molecules such as CK2 or early G1 cyclins may not have been activated. In fact, several investigators have proposed that $TGF\beta_1$ exerts its growth inhibitory effects during G1 stage of the cell cycle, without affecting the events of G0-G1 transition (Chambrad and Pouyssegur, 1988; Ko et al., 1994; Kletsas et al., 1995; Schoecklman et al., 1997). As indicated in the previous paragraph, it is also possible that $TGF\beta_1$ may have simultaneously affected early G1 cyclin-cdk complexes to prevent further progress of HPMC through G1. Since, EGF and $TGF\beta_1$ act as positive and negative regulators, respectively, to determine whether HPMC proliferate or not, and as this decision is made in G1, G1 cyclin-cdk may act as integrators of these extracellular signals (Polyak, 1996). These possibilities need to be examined further to clarify the mechanism by which EGF and $TGF\beta_1$ interactions regulate the proliferation of embryonic palate mesenchymal cells.

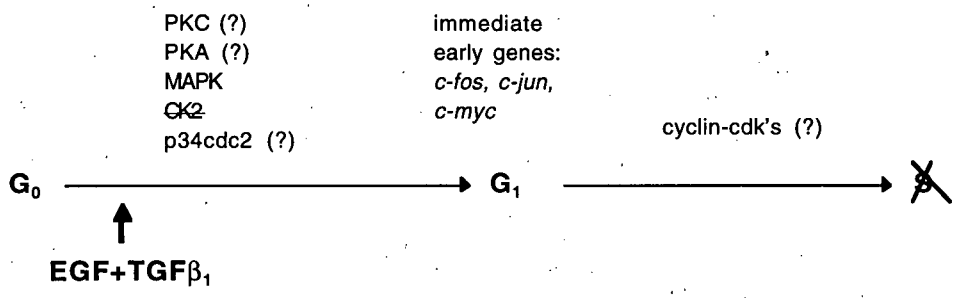


Figure 28. Effects of EGF+TGFβ_1 on signaling molecules and immediate early genes as palate mesenchymal cells advance from G0 through G1 phase of the cell cycle.

In summary, the results of the present study indicate that EGF and TGFβ_1 are important regulators of embryonic HPMC proliferation. Further, this study suggests that interaction among extracellular growth factors leads to modulation of the nuclear events that may be important in regulation of HPMC proliferation during palate morphogenesis.

The present study was undertaken on the overall premise that proliferation, differentiation, and death represent alternative and mutually exclusive pathways for cells during embryogenesis. There is a compelling notion in the literature that proto-oncogenes may function at critical control points in decision-making processes that regulate the biological phenomena. Hence, an objective of this study was to examine whether proto-oncogenes are expressed in HPMC as the cells commit themselves to DNA synthesis (i.e. cell proliferation). In addition, how EGF and TGFβ_1, implicated in regulation of cell proliferation during embryonic secondary palate development, modulate the activities of immediate early genes as the palate mesenchymal cells move from G0 to G1 phase of the cell cycle, was examined. This study provides hitherto unavailable information on how growth factor interaction could regulate the putative biological behavior of embryonic palate mesenchymal cells through modulation of the activities of immediate early genes. Clearly, a progress in understanding the proliferative/anti-proliferative mode of actions of extracellular factors can be achieved once activation of various other genes involved in cell cycle regulation, their interaction with inducing agents (such as growth factors) and with the components of basic

cell cycle machinery including signaling cascades, are thoroughly evaluated. As analyzed in Introduction of this thesis, there is a paucity of reports in the field of the developmental biology where regulation of proto-oncogene activity by multi-growth factor treatment has been analyzed in the primary culture of embryonic cells. From the perspectives of general cell and molecular biology, so far, much of the information on growth factor-regulated mechanisms that modulate functional behavior of cells has been derived from studies on transformed or established cell lines. Since these cell lines may have undergone sufficient mutations or phenotypic alterations, the application of information derived thence from to morphogenesis of a structure such as palate may prove to be complex and difficult. The approach outlined in the present study on the interactive effects of growth factors in primary culture of cells derived from embryonic palate mesenchyme, whose life history is fairly well defined, provides a potentially rewarding strategy to further analyze the regulatory cellular and molecular mechanisms in developmental biology, including those of normal and cleft palate formation.

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