COMMUNICATION OF SIGNALS AND RESPONSES LEADING TO CELL SURVIVAL/CELL DEATH USING ENGINEERED REGULATORY NETWORKS

Thesis submitted in fulfillment of the requirements for the Degree of

DOCTOR OF PHILOSOPHY

By

SHRUTI JAIN

Enrollment No. 086005

ELECTRONICS AND COMMUNICATION ENGINEERING
JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY,
WAKNAGHAT, SOLAN 173234
INDIA

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DECLARATION BY THE SCHOLAR

I hereby declare that the work reported in the Ph.D. thesis entitled **"COMMUNICATION OF SIGNALS AND RESPONSES LEADING TO CELL SURVIVAL/CELL DEATH USING ENGINEERED REGULATORY NETWORKS"** submitted at **Jaypee University of Information Technology,Waknaghat India,** is an authentic record of my work carried out under the supervision of **Dr. Pradeep K. Naik and Prof. (Dr.) Sunil V. Bhooshan**. I have not submitted this work elsewhere for any other degree or diploma.

(Shruti Jain)
Department of Electronics and Communication Engineering
Jaypee University of Information Technology, Waknaghat , India
May 2012

Jaypee University of
Information Technology

Waknaghat-173 215
Solan, Himachal Pradesh
Fax No.: 91-1792-245362

CERTIFICATE

This is to certify that the thesis entitled "**COMMUNICATION OF SIGNALS AND RESPONSES LEADING TO CELL SURVIVAL/CELL DEATH USING ENGINEERED REGULATORY NETWORKS**" submitted by **Mrs. Shruti Jain** at **Jaypee University of Information Technology, Waknaghat** in fulfillment of the requirement for the award of the degree of Doctor of Philosophy in Electronics and Communication Engineering is a record of bona fide research work carried out by her under our guidance and supervision and no part of this work has been submitted for any other degree or diploma

(Dr. Pradeep K. Naik)
Associate Professor
Biotechnology & Bioinformatics

(Dr. Sunil V. Bhooshan)
Professor
Electronics & Communication

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The great desire to acquire higher qualifications and pursue research drove me to promise my parents, teachers, brothers and my sister a useful research work. My promise was to live up to their expectations and never to let them down. And I kept my word.

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(*Shruti Jain*)

LIST OF ABBREVIATIONS

AIF	Apoptosis inducing factor
ANN	Artificial Neural Network
AP-1	Activation Protein 1
APAF-1	Apoptotic proteaseactivating factor-1
ASK1	Apoptosis signal-regulating kinase 1
BH	Bcl-2 homology domain
BiCMOS	Bipolar Complementary metal oxide semiconductor
cAMP	Cyclic adenosine monophosphate
CARD	Caspase-activating recruitment domain
Caspase	Cysteinyl aspartate-specific proteinases
CC	Caspase Cleavage
C. Elegans	Caernorhabditis elegans
CMOS	Complementary metal oxide semiconductor
CRADD	Caspase and RIP adaptor with death domain
CREB	CAMP response element binding protein
DED	Death effector domain
DISC	Death-inducing signaling complex
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ERK	Extracellular signal regulated protein kinase
FADD	Fas-Associated protein with Death Domain
Fas L	Fas ligand
FKHR	Forkhead transcription factor
FLIP	FADD-like ICE inhibitory protein
Grb2	Growth factor receptor-bound 2
GSK 3	Glycogen synthase kinase 3
HOG	High osmolarity glycerol
IAP	Inhibitor of Apoptosis
IGF	Insulin-like growth factor

IGF1R	Type 1 insulin-like growth factor receptor
IGF2R	Type 2 IGF receptor/ mannose-6-phosphate receptor
IKK	IκB kinase
IR	Insulin receptor
IRS1	Insulin receptor substrate 1
IκB	I Kappa B (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor)
JNK	c-jun NH_2 terminal kinase
MAP	Mitogen activated protein
MAPK	Mitogen activated protein kinase
MATLAB	Matrix Laboratory
MEK	Mitogen-activated protein kinase and extracellular-regulated kinase kinase
MEKK	Mitogen-activated protein kinase / ERK kinase kinase-1
MK2	Mitogen-activated protein kinase-activated protein kinase 2
MP	Membrane Permeability
mTOR	Mammalian target of rapamycin
NF	Nuclear fragmentation
NF-κB	Nuclear factor kappa B
p38	P38 mitogen-activated protein kinases
PARP	poly (ADP-ribose) polymerase
PDK	3-phosphoinositide dependent kinase
PDK	Phi Delta Kappa
PE	Phosphatidylserine exposure
PI-3K	Phosphatidylinositol-3- kinase
PLC	Phospholipase C
PTEN	Phosphatase and tensin homolog deleted on chromosome ten
PTK	Protein tyrosine kinase
Rac	Ras-related C3 botulinum toxin substrate
RAIDD	RIP associated Ich-1/CED homologous protein with death domain
SODD	Silencer of death domains

SOS	Son of Sevenless
SPICE	Simulation program for integrated circuit emphasis
TGF	Transforming growth factor
TNF	Tumor necrosis factor
TNFR1	Tumor necrosis factor receptor 1
TNFR2	Tumor necrosis factor receptor 2
TRADD	TNF-receptor associated death domain
TRAF2	TNF receptor associated factor 2
TSC	Tuberous sclerosis complex
XIAP	X-linked Inhibitor of Apoptosis Protein.
VHDL	VHSIC Hardware Description Language

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ABSTRACT

Biological systems can create complex structures from very simple systems. To do this, there must be a method to differentiate different regions where identical systems create different structures, such as the abdomen and the head of a fruit fly. This thesis highlights an emerging field known as *Synthetic Biology* that envisions integrating designed circuits into living organisms in order to instruct them to make logical decisions based on the prevailing intracellular and extra cellular conditions and produce a reliable behavior. Synthetic Biology attempts to construct and assemble such modules gradually, plug the modules together and modify them, in order to generate a desired behavior. Using biological circuit, we can produce a new concentration gradient that has twice the frequency. If '1' is represented by a concentration of the chemical within the threshold, and a '0' is represented by a concentration outside the threshold, then we can represent any two digit binary number. Thus, we can differentiate separate regions at certain distances away from a point source. Logic gates are the basic building blocks in electronic circuits that perform logical operations. These have input and output signals in the form of 0's and 1's; '0' signifies the absence of signal while '1' signifies its presence.

The study focuses on system implementation of a signals and responses triggered by Tumor Necrosis Factor- α (TNF-α), Epidermal Growth Factor (EGF) and Insulin factors leading to cell survival/ apoptosis. The computational techniques that have been used are: VHDL (Xilinx Tool), CMOS and BiCMOS (SPICE Tool), and Fuzzy Logic (MATLAB Tool), Non Linear Model (Artificial Neural Networks) and Deterministic Model taking three input signals. In future we are also trying to develop bio-simulator that can be used for prediction of status of the cell leading to cell survival/ apoptosis giving chemotherapy and radiotherapy treatment.

We have presented an integrated theoretical framework for describing the balance between cell survival/ apoptosis regulated by TNF, EGF and insulin. The theoretical models consider here for mathematical intervention is minimal in the sense it takes into consideration only those reactions that are essential to describe the action of TNF, EGF and Insulin on cell survival/ apoptosis. We have modeled TNF, EGF and Insulin binding and its uptake by cells, and it is worth noting that the essential parameter values are biologically relevant. The results illustrated

that the rate constants defined in the deterministic models are biologically relevant of key proteins in the signaling pathway which decide the fate of cell. The quality of fit between theoretical and experimental values has been represented. The minimum root mean square error (RMSE value) between theoretical and experimental values for the marker proteins revealed good accuracy of the model.

We have extensively explored the parameter space of the model. Simulations show that the model has a stable behavior for a broad range of parameter values and that no unexpected patterns emerge (such as oscillations, chaos, etc.). Thus the model is structurally stable: obviously this is quite important and lends credibility to the model, because this indicates that different cells with unequal expression of key substrates and enzymes and/or showing a signal transduction network with a different topology have similar responses to TNF, EGF and Insulin. This also means that our model is not specific for a given cell type and that it can be used to simulate the effects of TNF,EGF and Insulin independently for the experimental settings of the original data on which the parameter estimate is based. In our model there is no switching mechanism that selects cell survival/cell death signals, but rather a balance between the two pathways that produces partial cell killing even for long lasting and intense TNF, EGF and Insulin treatments. The balance depends on environmental TNF, EGF and Insulin concentration, and this observation might be important to explain cellular homeostasis during an immune response, i.e. the fine equilibrium between cellular activation and death. This equilibrium might further be balanced in favor of cell survival or death in real cells by the fine expression and/or degradation of intracellular molecular actors that transduce TNF, EGF and Insulin signals.

CHAPTER 1
INTRODUCTION

Computational Biology has recently emerged at system-level understanding of biological processes. Biological signaling networks process extracellular cues to control important cell divisions such as survival -death, growth-quiescence, and proliferation-differentiation [1]. Communication between the response of cells with extracellular signals such as cytokines, growth factors, and hormones is mediated by receptors that transduce cellular cues into changes in intracellular physiology. Downstream of receptors, signal communication networks [2, 3] are controlled by large sets of proteins acting in concert. In case of programmed cell death, *Tumor necrosis factor- α* (TNF-α) [4] functions as cell death cues, whereas growth factors such as Epidermal growth factor (EGF) [5, 6] and insulin [7, 8, 9] exert survival effects. The magnitudes of the responses vary with cell type, but the pathways downstream of cytokine receptors are conserved and highly interconnected. It appears that the determination of whether a cell will live or die involves a balance between cell survival/cell death [10, 11, 12]. Thus, the intracellular signal communication network stimulated by TNF, EGF and insulin acts as a signal processor that converts opposing cues into a functional response that controls cell fate. Moreover, the complexity of cellular communication networks precludes a simple protein-by-protein assignment of function.

Increasingly, systematic methods are being applied to the interpretation and computational analysis of cell signaling [13]. These methods are useful for codifying existing prior knowledge in pursuit of in-silico predictions. The signal processing pertaining to cell survival/cell death are ripe for applying various computational techniques to tease out the key biochemical changes associated with critical cell decisions because of the availability of experimental data. However, the experimental data that have been available are from the measurement of a wide range of parameters including protein abundance, localization, enzymatic activity, and post translational modification. In addition, protein measurements involve a variety of techniques, including western blots, kinase assays, protein microarrays, and imaging. The heterogeneous nature of the

data for cell signaling studies present a challenging problem for data integration into a single coherent model.

There are numbers of exciting and profound issues that are actively investigated, such as robustness of biological systems, network structures and dynamics and applications to drug discovery. Systems biology is in its infancy, but this is the area that has to be explored and the area that we believe to be the main stream in biological sciences in this century [13]. Systems biology aims to explain how higher level properties of complex biological systems arise from the interactions among their parts.

The goal of Synthetic Biology is to extend or modify the behavior of organisms and engineer them to perform new tasks. One useful analogy to conceptualize both the goal and methods of Synthetic Biology is the computer engineering hierarchy. Within the hierarchy every constituent part is embedded in a more complex system that provides its context. Designing of new behavior occurs with the top of the hierarchy in mind but is implemented bottom-up. At the bottom of the hierarchy shown in Fig 1.1 are DNA, RNA, proteins and metabolites (including lipids and carbohydrates, amino acids, and nucleotides), analogous to the *physical layer* of transistors, capacitors and resistors in Computer and Electronics engineering. The next layer, called the *device layer*, comprises biochemical reactions that regulate the flow of information and manipulate physical processes is equivalent to engineered logic gates that perform computations in a computer. At the *module layer*, the Synthetic Biologist uses a diverse library of biological devices to assemble complex pathways that function like integrated circuits. The connection of these modules to each other and their integration into host cells allows the synthetic biologist to extend or modify the behavior of cells in a programmatic fashion. Although independently operating engineered cells can perform tasks of varying complexity, more sophisticated coordinated tasks are possible with populations of communicating cells, much like the case with computer networks.

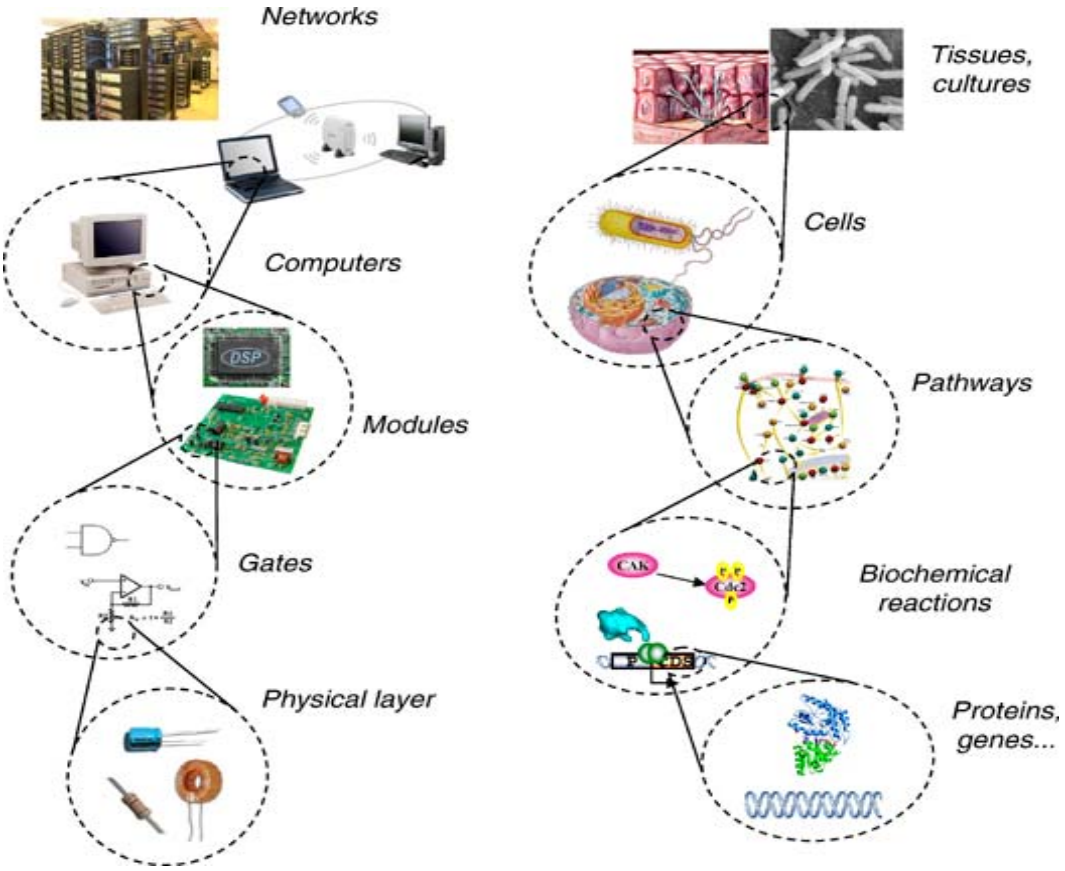


Fig. 1.1 Hierarchy of Electronic and Biological Elements

1.1 APOPTOSIS OR CELL DEATH

The term *cell death/ programmed cell death* was coined in the early 1970, although the phenomenon of cell death was already described in the late 1700s. The word, cell death, is derived from Greek roots meaning, "Dropping off" e.g. falling of leaves. It plays an important role in the development and maintenance of tissue homeostasis but it also represents an effective mechanism by which abnormal cells, such as tumor cells, can be eliminated [12, 14]. Although the apoptotic destruction itself is an "expensive" process that consumes much energy and building materials it is a sound investment in terms of the organism as an entity. When compared to the life of the whole organism, cells are apparently cheap and expendable. The cell death process can be divided into at least three functionally distinct phases: *initiation*, *effector* and *degradation*. During the heterogeneous initiation phase cells receive death-inducing signals: lack of obligatory

survival factors, shortage of metabolite supply and ligation of death-signal transmitting receptors, sub necrotic damage by toxins, heat or irradiation. During the effector phase, these signals are translated into metabolic reactions and the decision to die is taken.

1.2 PHASES OF CELL DEATH

It has been shown that cell death is triggered by different intracellular and extra cellular stimuli and proceeds in two phases: an initial *commitment phase* and an *execution phase* shown in Fig 1.2, which results in typical apoptotic morphological changes in the cells such as plasma membrane blebbing, shrinkage of the cytoplasm, dilation of endoplasmic reticulum, nuclear chromatin condensation and fragmentation into apoptotic bodies that are phagocytosed by neighboring cells. During cell death cells reduce their volume, pump out ions and there is contraction of the cytoskeleton, forming a cage-like structure around the nucleus. At the same time, the dying cells activate their own proteolytic enzymes in addition to those acting in the phagolysosomes.

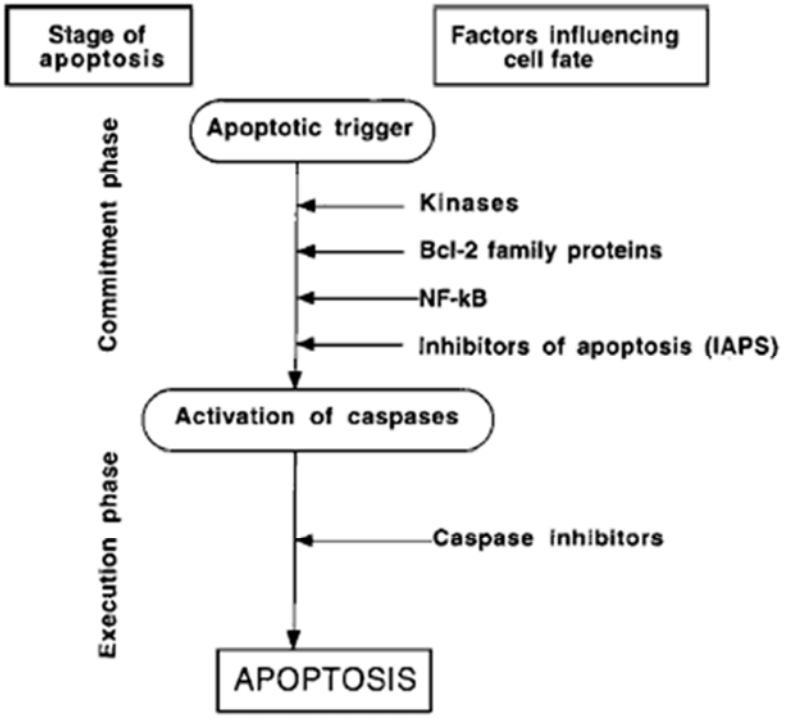


Fig. 1.2 The apoptotic pathway: The apoptotic pathway consists of a commitment phase, during which the fate of the cell is decided and an execution phase during which caspases are activated and mediate cell death. Many factors determine the influence of the cell survival or death. All these factors represent potential targets of therapeutic intervention that can be used to promote or prevent cell death.

During the first phase, called the "*commitment phase*", an individual cell "decides" whether or not to enter the execution phase and die or remain viable. The ultimate result of exposure to an apoptotic trigger, life or death, is determined by a balance between a number of pro- and anti-apoptotic pathways present in every cell. If a cell is destined to die, the second stage of cell death, called the "*execution phase*" is activated. The execution of cell death entails the controlled activation of a number specific effector mechanisms that lead to the classic morphologic features of cell death described above.

1.2.1 THE EXECUTION PHASE OF THE APOPTOTIC PATHWAY

Role of caspases/ interleukin-1b-converting enzymes (ICE): Caspases, the family of proteases are largely responsible for the execution phase of cell death [15]. The caspases are present in cells in an inactive form (pro-caspase) and form a tightly regulated, sequential, and self-amplifying cascade. Caspases are responsible for almost all the biochemical and morphologic features of cell death and act by the proteolytic cleavage of a host of cellular proteins. All caspases share a number of structural and functional features. The "*c*" refers to the fact that caspases are cysteine proteases, with the catalytic site cysteine contained within a conserved QACXG motif (single letter amino acid code), whereas the "*aspase*" refers to the unique and absolute predilection of all caspases for cleaving proteins after aspartic acid residues. While it is not yet possible to provide a complete flow diagram of the precise cascade of reactions by which caspases mediate the apoptotic pathway, caspases can be divided into two main functional classes; "*initiator*" caspases and "*effector*" caspases. Caspase-mediated cleavage of cell death-specific endonuclease CAD (caspase-activated DNase) is responsible for the "ladder" pattern of DNA fragmentation typical of cell death. Caspases also are responsible for proteolysis of the nuclear lamins thereby, facilitating nuclear condensation. Caspases that target cyto skeletal proteins such as α-fodrin, β-actin, and keratins mediate disassembly of the cell cytoskeleton [16]. Other classes of caspase substrates cleaved during cell death include DNA repair enzymes, signal transduction molecules as well as transcriptional and cell cycle regulators.

Mechanisms of caspase activation

In general, there are several ways by which caspases can be activated. The **first** is as a result of cleavage by autolysis and by upstream proteases, such as other caspases or granzyme B (GrB), which is the only mammalian serine protease that shares the caspase specificity for the Asp residue at the P1 position. A common feature of the ICE family proteases is the presence of a pro domain that has been hypothesized to keep the enzyme in an inactive form. Pro domain is also necessary for dimerization, which occurs prior to auto processing. GrB in vivo processes initially caspase-3, which in turn can remove the pro domain from caspase-7, and, finally, caspase-7 is fully processed by GrB. In a heterologous expression system it has been shown that recombinant caspase pro enzymes are autolytically processed to their mature forms when synthesized at sufficiently high levels. A mutation in catalytic cysteine residue prevents this processing. Isolated caspase pro enzymes can be auto activated when they are concentrated by ultra filtration .

A **second mechanism** of caspase activation through the '*extrinsic*' pathway is initiated by triggering cell death receptors on the cell surface, leading to activation of the intracellular apoptotic machinery (death signal-induced, death receptor-mediated pathway). Cell death signals, such as Fas ligand (FasL) and tumor necrosis factor (TNF)-2, can be specifically recognized by their corresponding death receptors, such as Fas or TNF receptor (TNFR)-1, in the plasma membrane [17]. Their binding will in turn activate the death receptors. Fas can bind to the Fas-associated death domain (FADD) (or TNFR-associated death domain, TRADD) and cause FADD aggregation and the emergence of DEDs. These exposed DEDs interact with the DEDs in the pro domain of procaspase-8, which will induce the oligomerization of procaspase-8 localized on the cytosolic side of the plasma membrane. Then a massive molecule complex known as the death-inducing signal complex (DISC) is formed. This complex is called DISC (death-inducing signaling complex), and it is thought that as more procaspase-8 molecules become involved in this complex, they are activated, probably by auto cleavage. Similar mechanisms have been demonstrated for the tumor necrosis factor receptor (TNF-R1), which seems to require an additional adapter molecule TRADD (TNFR-associated death domain), which recruits FADD and procaspase-8. Furthermore, TRADD can recruit serine-threonine kinase RIP

(receptor interacting protein) and an adapter molecule RAIDD, which has sequence similarity with the pro domains of caspases-2, -9 and CED-3 and can activate procaspase-2. At the same time, the physiological role of caspase-2 in the activation cascade of caspases remains unclear. It is likely that caspases-8 and -10 are the major proteases in TNF and anti-Fas induced cell death, which activate the downstream caspases. In the next step, activated downstream caspases can activate themselves and other caspases, leading to the amplification cascade of caspase activation. The recruitment of caspases-8 and -10 may be inhibited by viral inhibitors of the apoptotic signals (FLIPs). These inhibitors contain two N-terminal DEDs in their structure. They interfere with FADD-caspase-8 binding and inhibit both caspase-8 activation and cell death. Caspases -1, -2, -4, -5, -9 and CED-3 contain another "caspase recruitment domain" (CARD), required for assembly of activation complexes [15]. The activation of the downstream pathways of caspase-8 varies with different cell types (Fig. 1.3).

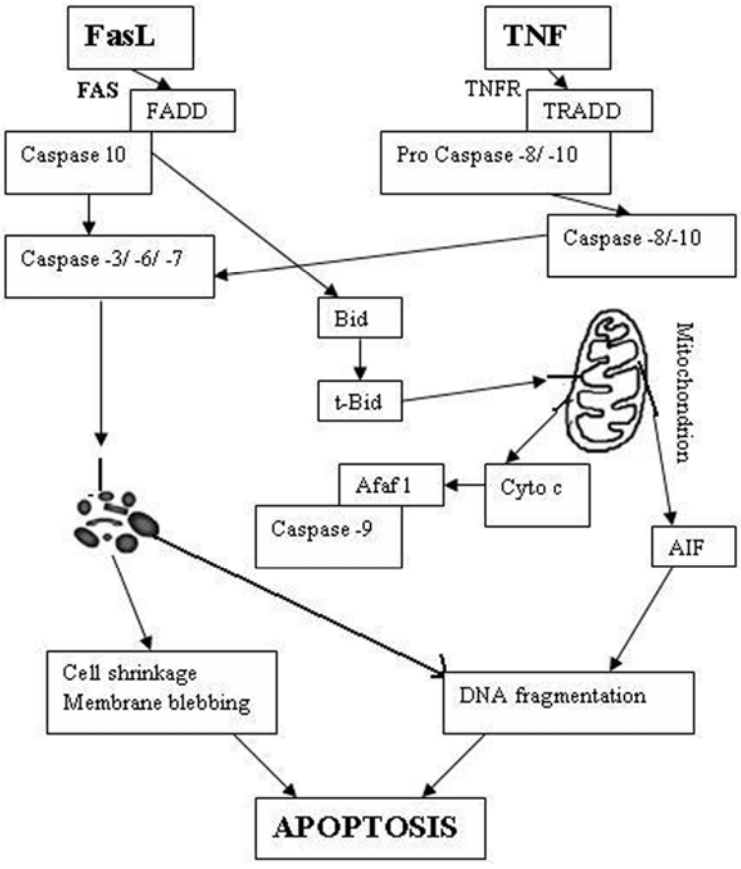


Fig. 1.3 Caspase-8/caspase-10-dependent procaspase-activation pathway

In Type I cells (cells of some lymphoid cell lines), caspase-8 is vigorously activated and can directly activate the downstream pro caspases (e.g. procaspase-3). In Type II cells (other than Type I cells), caspase-8 is only mildly activated and unable to activate procaspase-3 directly [18, 19]. However, it can activate the mitochondrion-mediated pathway by truncating Bid (a pro-apoptotic Bcl-2 family member), a kind of pro apoptotic protein in the cytosol, into its active form, tBid. tBid will trigger the activation of the mitochondrion pathway: cytochrome c, cell death-inducing factor (AIF) and other molecules are released from mitochondria, and cell death will be induced.

The activation pathway mediated by procaspase-10, with a DED-containing prodomain, is similar to that mediated by procaspase-8. Caspase-10 functions mainly in the cell death of lymphoid cells. It can function independently of caspase-8 in initiating Fas- and TNF-related cell death. Moreover, Fas cross linking in primary human T cells leads to the recruitment and activation of procaspase-10. Although caspase-8 and caspase-10 both interact with the DED of FADD in death receptor signaling, they may have different cell death substrates and therefore potentially function distinctly in death receptor signaling or other cellular processes.

The **third way** in which the caspase cascade can be initiated involves translocation of cytochrome c from the *mitochondria* to the cytoplasm (non-receptor-mediated pathway or stress-induced cell death) (Fig. 1.4). The mechanism of this translocation remains unclear and may be due to opening of a mitochondrial permeability transition pore, rupture of the outer membrane or the presence of specific channels for cytochrome c [18, 19]. Cytochrome c release from mitochondria is under the control of the Bcl-2 family of proteins, [20] that either inhibit (Bcl-2, Bcl-xL) or promote (Bax, Bak, Bik, Bid) cell death. In the cytoplasm, cytochrome c interacts with Apaf-1 (the human homolog of C. elegans protein CED-4) [21]. The '*intrinsic*' pathway (Mitochondrion-mediated pro caspase-activation pathway) of cell death is initiated via the mitochondria by cellular stress, such as chemotherapeutic drugs and radiation (the stress-induced, mitochondrion-mediated pathway) (i.e. a caspase-9-dependent pathway). The elucidation of the molecular mechanisms regulating these processes is of primary interest.

a) *Mitochondrion-mediated pro caspase-activation pathway of caspase-8* : Apart from being recruited to form a DISC complex after auto activation, procaspase-8 could also be activated through a cytochrome c-dependent pathway. After cytochrome c is released from mitochondria to the cytosol, caspase-6 is the only cytosolic caspase with the ability to activate procaspase-8, which depends solely on procaspase-6 activation by pro domain cleaving. It means that, in the cytochrome c-dependent pathway, the activation of procaspase-8 requires neither the interaction with FADD nor the formation of a DISC complex.

b) *Mitochondrion-mediated pro caspase-activation pathway of caspase-9* : When cellular stress (e.g. DNA damage) occurs, pro apoptotic proteins in the cytosol will be activated, which will in turn induce the opening of mitochondrion permeability transition pores (MPTPs) [22]. As a result, cytochrome c localized in mitochondria will be released to the cytosol. With the presence of cytosolic dATP (deoxyadenosine triphosphate) or ATP, apoptotic protease activation factor-1 (Apaf-1) oligomerizes [23]. Together with cytosolic procaspase-9, dATP and cytochrome c, oligomerized Apaf-1 can result in the formation of a massive complex known as apoptosome. They interact with each other by CARDs and form a complex in the proportion of 1:1 [24]. Activated caspase-9 can in turn activate procaspase-3 and procaspase-7. The activated caspase-3 will then activate procaspase-9 and form a positive feedback activation pathway shown in Fig 1.4. Fig 1.5 shows the combined Intrinsic and Extrinsic Pathway.

1.2.2 THE COMMITMENT PHASE OF APOPTOPSIS

After exposure to an apoptotic trigger, a cell enters phase of variable duration during which the fate of the cell, cell death or survival, is decided. This is called the commitment phase of cell death. The outcome of the commitment phase depends upon the balance of a number of different factors, some of which are anti-apoptotic and others that promote precipitation of the execution phase of cell death.

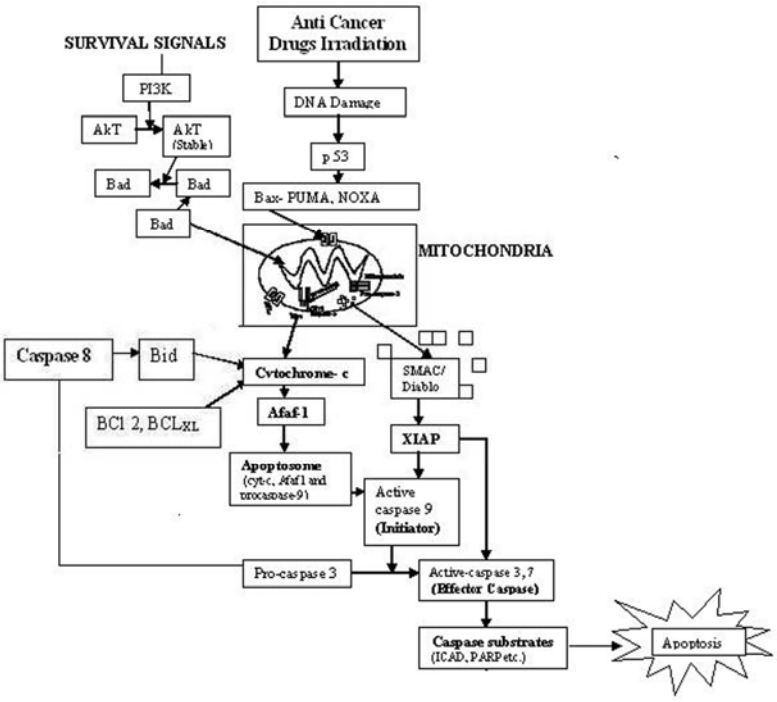


Fig. 1.4 The intrinsic pathway of cell death triggered by TNF and mediated by mitochondria.

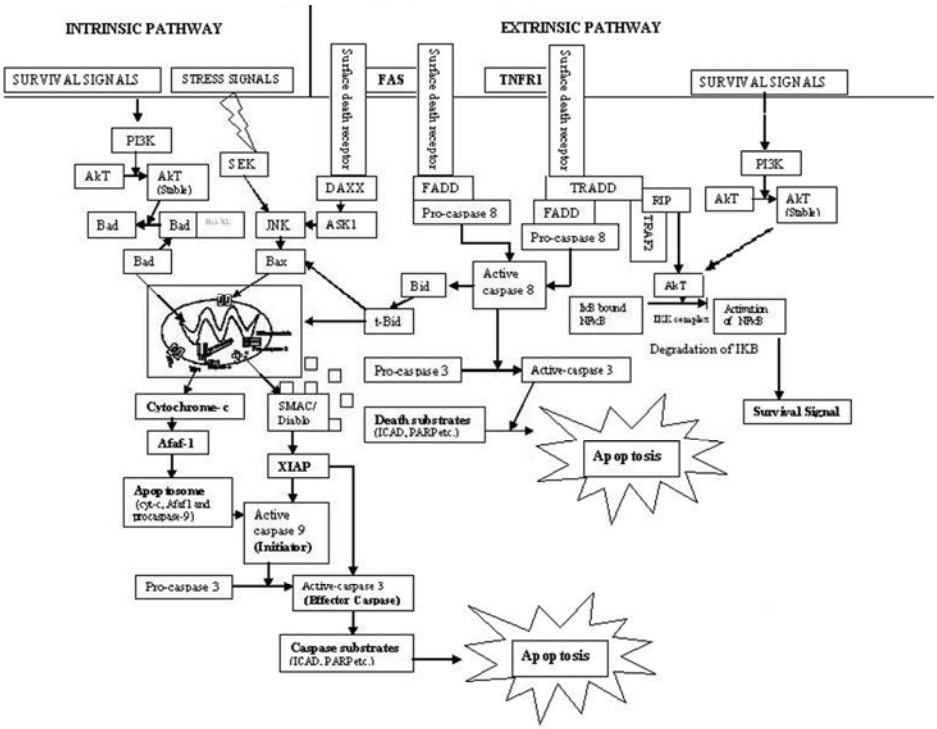


Fig. 1.5 Schematic representing the core components of cell death pathways. In the extrinsic pathway, TNF super family members including Fas Ligands binding to a death receptor and forming a death inducing Signaling complex (DISC), which activate caspase-8. In the intrinsic pathway, cytochrome c released from mitochondria causes apoptosome formation and caspase-9 activation. Both caspase-8 and caspase-9 activate down stream caspases like caspase-3 and leading to cell death.

Bcl-2 family of proteins :

The first member of this family, proto-oncogene bcl-2, was originally found at the breakpoints of t(14;18) chromosomal translocation in low-grade B cell lymphomas. The bcl-2 gene was thought to be a unique oncogene that contributed to cell expansion through failed cell death rather than rapid cell division [25]. However, it is now known that many cancers express high levels of bcl-2 without evident gene alterations, suggesting that other pathways also contribute to bcl-2 expression. The bcl-2 protein seems to be multifunctional since it is able to hetero-dimerize its pro-apoptotic relative bax, to bind to non-homologous proteins and to form ion-channels. Bax (Bcl-2 homologous antagonist x) is a bcl-2 related protein that promotes cell death and acts as a tumor suppressor [26, 27]. Bax is thought to be a downstream transcription target of p53 and may thus play a part in p53 apoptotic pathway. There is evidence that the ratio of bcl-2 to bax determines the susceptibility of a cell to cell death. When over expressed, bax forms homo dimers, thereby accelerating cell death. In contrast, when bcl-2 is expressed in excess, it hetero dimerises with bax and cell death is suppressed. Consistent with its tumor suppressor role in human cancer, bax has been shown to be mutated or expressed at a reduced level in several human cancers, including colon and breast cancer as well as haematopoietic malignancies. The expression of bax is predominant in large cell neuro endocrine lung carcinoma and inversely associated with bcl-2 expression.

Bcl-2 and related proteins can be divided on the functional basis into two distinct groups, those with anti-apoptotic activity and those that promote cell death. Pro-survival members of this family can inhibit cell death induced by an extremely wide range of triggers, including survival factor deprivation. Bcl-2 family members play an important role in influencing cell fate during the commitment phase of cell death. Bcl-2 family members bind to one another to form hetero-dimers. Dimerization of Bcl-2 members with opposite effects on cell death results in a titration of the effects of each of the interacting proteins. In addition to direct interaction, Bcl-2 proteins may influence the effects of other members by competing for common downstream targets. Thus, the outcome of the commitment phase appears to depend, at least in part, on the relative concentrations of pro-survival and pro-apoptotic Bcl-2 family members. Bcl-2 proteins appear to regulate cell death in two major ways. The *first* involves the direct interaction of Bcl-2 family

members with procaspase activating complexes. Activation of procaspase-9 occurs within a so-called “apoptosome”, in which cytoplasmic Apaf-1 binds via separate domains to procaspase- 9 and cytochrome c Pro-survival Bcl-2 family members, such as Bcl-xL, by binding to Apaf-1, can inhibit the cytochrome c-induced change in Apaf-1 that leads to the recruitment and activation of procaspase-9 [28]. Thus, pro-survival Bcl-2 family members may block initiation of the execution phase of cell death, even after mitochondrial permeability transition and release of cytochrome c has occurred. Pro-apoptotic Bcl-2 family members may counteract the protective effects of Bcl-xL by binding to and sequestering Bcl-xL [29]. The *second* mechanism of action of Bcl-2 family members involves alterations in the permeability of mitochondrial membranes. In response to several triggers of cell death, pro-survival proteins Bcl-2 or Bcl-xL stabilize the mitochondrial membrane, thereby inhibiting mitochondrial permeability transition and the release of cell death promoting substances such as cytochrome c and AIF. It is still uncertain how Bcl-2 and Bcl-xL stabilize the mitochondrial membrane. In any case, these proteins protect cells by acting at two discrete but sequential steps in the apoptotic pathway; by inhibiting the release of mitochondrial cytochrome c, and once cytochrome c is released, by interfering with cytochrome c-mediated activation of procaspase 9 [30, 31]. In contrast to the stabilizing effects of pro-survival Bcl-2 on the mitochondrial membrane, some pro-apoptotic proteins such as Bax, and Bid directly induce mitochondrial permeability transition and the release of cytochrome c. These proteins appear to act by inserting into the mitochondrial membrane and forming pores and ion conducting channels.

The Bcl-2 proteins have a role both in the commitment phase and the execution phase of cell death. During the commitment phase, Bcl-2 proteins are regulated primarily via phosphorylation events. Phosphorylation events can increase or decrease the activity of pro-survival proteins such as Bcl-2 and Bcl-xL. On the other hand, phosphorylation of the pro-apoptotic member BAD by PKB/Akt leads to the sequestration of BAD within the cytosol, thereby preventing access of BAD to mitochondria where it can hetero dimerize with and inactivate pro survival Bcl-2 family members [32]. During the execution phase, the activity of these proteins can be altered by caspase-mediated cleavage. In some cases, caspase-mediated cleavage can convert proteins from pro-

survival to pro-apoptotic activity. Thus, cleavage of Bcl-2 abrogates its pro-survival activity and converts the protein into a Bax-like pro-apoptotic factor. Also, caspase-8 activates the pro-apoptotic protein Bid, and converts it from a latent cytoplasmic form to an active moiety which moves to mitochondria and promotes the release of cytochrome c. These examples highlight the complexity of regulation not only among Bcl-2 family members but also between Bcl-2 family members and other components of the apoptotic machinery.

NF-κB: A powerful anti-apoptotic transcription factor: The term NF-κB refers to a family of nuclear transcription factors that regulate the transcription of genes involved in the immune response and cell death. It has become evident that activation of NF-κB plays an important role in opposing cell death and therefore in determining cell fate in response to a number of apoptotic triggers [33]. When inactive, NF-κB is present within the cytoplasm complexed to one of several inhibitory proteins known collectively as IκB, which prevent NF-κB from entering the nucleus. An extremely wide range of stimuli, including some apoptotic triggers, activates NF-κB-Inducing Kinase (NIK) which phosphorylates I*κ*B, thereby leading to its rapid proteasomal degradation. This allows the active NF-κB to translocate to the nucleus where it binds to specific motifs in the promoter regions of its multiple target genes. The anti-apoptotic effect of NF-κB was first clearly demonstrated for TNF-α. TNF-α *binds* with high affinity to two distinct receptors, TNF-R1 and TNF-R2, with opposing effects on cell fate. The death receptor TNFR1 induces cell death through recruitment and activation of the death domain-containing proteins TRADD and FADD, ultimately leading to the activation of the apoptotic initiator pro caspase 8. By contrast, signaling through TNF-R2, which lacks a death domain and so does not activate TRADD and FADD, generally promotes survival and proliferation. However, the division in signaling pathways induced by TNF-R1 versus TNFR2 is not absolute. NF-κB is activated via engagement of both receptors. The strong induction of NF-κB by both TNF-R1 and TNF-R2 accounts for the fact that induction of cell death by TNF-α generally requires the concomitant addition of a protein synthesis inhibitor such as cyclo heximide [34, 35]. NF-κB protects against cell death in a number of ways. These include the transcriptional induction of various members of the IAP family of cell death inhibitors as well as of the anti-apoptotic Bcl-2 family member Bfl- A1; the

induction of a novel inhibitor of cell death, named IEX-1L; the inhibition of p53 activity through competition for a limiting shared co-factor; and an increase in the expression of TNF receptor-associated proteins such as TRAF2. It is likely that the contribution and magnitude of these pro-survival effects of NF-κB will depend on the cell type and inducing stimulus. It is important to note that the pro-survival effect of NF-κB is unlikely to be restricted to TNF-induced cell death since other stimuli, such as growth factors and oxidant stress are known to activate NF-κB. Thus NF-κB may turn out be potent anti-apoptotic response to a ubiquitous array of apoptotic triggers.

1.3 COMMUNICATION OF SIGNAL TRIGGERED BY TNF-α LEADING TO CELL SURVIVAL / CELL DEATH

Tumor necrosis factor (TNF) is a cytokine that mediates cell death, cell proliferation, inflammation, allergy, arthritis, septic shock, insulin resistance, autoimmune diseases, and other pathological conditions [36, 37]. TNF transduces these cellular responses through two distinct receptors: type I, which are expressed on all cell types, and type II, which are expressed only on cells of the immune system and endothelial cells. TNF binds with high affinity to two-cell surface receptors, a 55kd protein (p55TNF-R/ TNFR1) and a 75kd protein (p75TNF-R/ TNFR2), both are expressed by most cell lines and primary tissues [36]. At the cellular level, these receptors activate the pathways leading to the activation of transcription factors NF-kappa B and AP-1, cell death and proliferation, and mitogenic activated protein kinases.

TNF-R1 is constitutively expressed in most tissues, and can be fully activated by both the membrane-bound and soluble trimeric forms of TNF, while TNF-R2 is only found in cells of the immune system and respond to the membrane-bound form of the TNF homotrimer. As most information regarding TNF signaling is derived from TNF-R1, the role of TNF-R2 is likely underestimated. The binding of TNF-α to the TNF receptor type I (TNF-RI) promotes the recruitment of several intracellular adaptors which in turn, activate multiple signal transduction pathways. While recruitment of death domain (DD) containing adaptors such as Fas associated DD (FADD) and TNF-R associated DD (TRADD) [38] can lead to the activation of signal transduction pathways that induce cell

death, recruitment of TNF-RI associated factors (TRAFs) can lead to the activation of multiple cell survival intracellular signals such as NF-κB, JNK, p38 and ERK [39]. Signaling pathways of TNFR1 is shown in Figure 1.6. MAPK and IKK in turn activate AP-1 and NF-κB transcription factors. Activation of AP-1 and NF-κB induces genes involved in inflammation, immune response, cell proliferation and cell differentiation, as well as genes that act to suppress death receptor- and stress-induced cell death.

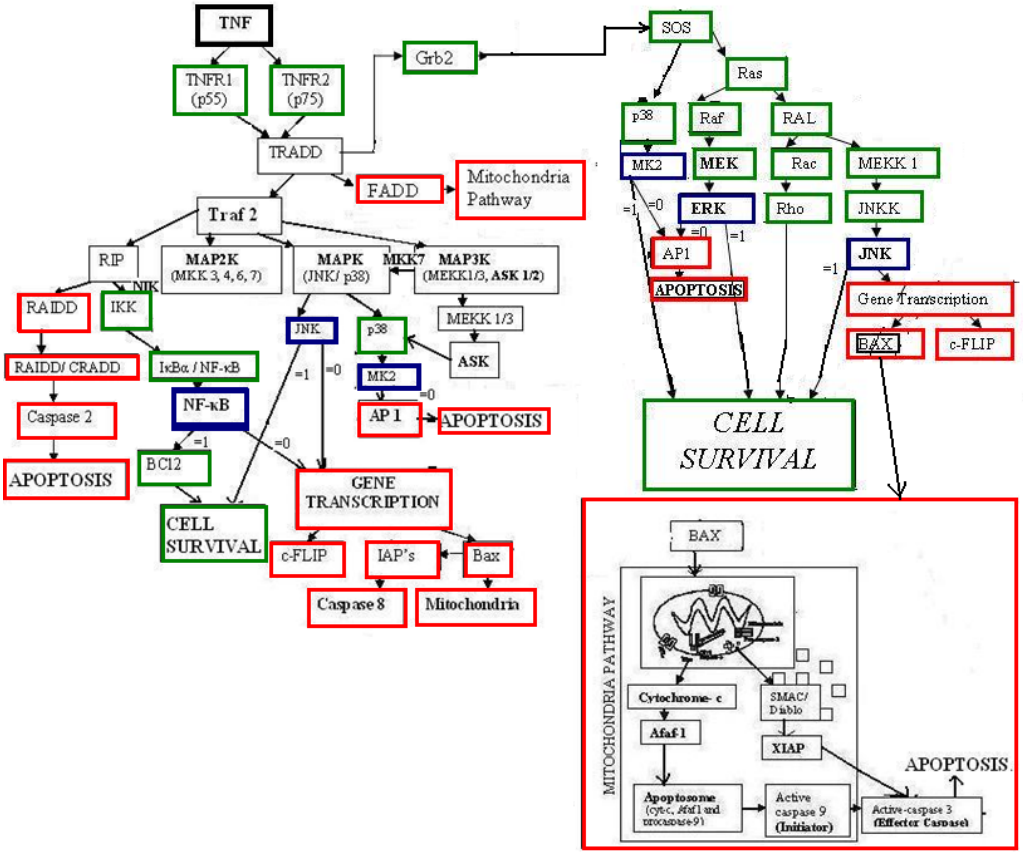


Fig. 1.6 Illustration of signal communication network triggered by TNF. The box marked in *red color* are the proteins involved in cell death pathway, the box marked in *green color* are the proteins involved in cell survival pathway and the box marked in *blue color* are the proteins involved in both cell survival/ cell death

The following TRADD binding pathways can be initiated as follows

1.3.1 Activation of NF-κB: NF-κB is widely used by eukaryotic cells as a regulator of genes that control cell proliferation and cell survival. Active NF-κB turns on the expression of genes that keep the cell proliferating and protect the cell from conditions

that would otherwise cause it to die. In tumor cells, NF-κB is active either due to mutations in genes encoding, the NF-κB transcription factors themselves or in genes that control NF-κB activity (such as IκB genes); in addition, some tumor cells secrete factors that cause NF-κB to become active. Blocking NF-κB can cause tumor cells to stop proliferating, to die, or to become more sensitive to the action of anti-tumor agents. Thus, NF-κB is the subject of much active research among pharmaceutical companies as a target for anti-cancer therapy. TRAF2 in turn recruits the multicomponent protein kinase IKK, enabling the serine-threonine kinase RIP to activate it. An inhibitory protein, IκBβ , that normally binds to NF-κB and inhibits its translocation, is phosphorylated by IKK and subsequently degraded, releasing NF-κB [34, 35]. NF-κB is a heterodimeric transcription factor that translocates to the nucleus and mediates the transcription of a vast array of proteins involved in cell survival and proliferation, inflammatory reponse, and anti-apoptotic factors. NF-κB is a family of transcription factors, which induce the expression of a wide variety of genes, especially those involved in survival, such as the Bcl-2 family member Bfl-1, and the caspase inhibitors c-IAP1 and c-IAP2.

1.3.2 Activation of the MAPK pathways: The MAPKs consist of several subfamilies such as ERK, JNK/SAPK (c-Jun amino-terminal kinase / stress-activated protein kinase), and p38/ MK2 [40, 41, 42]. They act in distinct and independent signaling pathways with a wide range of cellular responses including proliferation, differentiation and survival. The signaling through ERK 1 and 2 has a major role in the stimulation of cell proliferation; they have been shown to be translocated to the nucleus and induce gene expression that promotes the cell cycle entry. There is evidence of direct regulation of cell death by ERK (downstream of b-Raf) through cytosolic caspase inhibition.

The other MAPK members, p38 and JNK/SAPK represent signaling pathways homologous to the Ras-MAPK pathways, which are involved in the regulation of cellular responses to stress. These pathways, in contrast, are not activated primarily by mitogens but by various kinds of cellular stress instead and inflammatory cytokines, and result in cell death.

- **The RAS/ERK pathway :** Growth factor stimulation activates various signaling pathways that result in the induction of a variety of genes involved in the regulation of cell proliferation, cell differentiation and cell cycle progression. The ERK MAP kinase cascade is one of the central pathways in growth factor signal transductions. In response to growth factor stimulation, classical MEK becomes activated, and then the activated MEK phosphorylates and activates classical MAP kinase ERK. EGF/IRS activates the ERK pathway through the binding of Grb2 or Shc to phosphorylated ErbB receptors, which in turn results in the recruitment of the son of sevenless (SOS) to the activated receptor dimmer SOS then activates RAS leading to the activation of RAF 1 [43]**.** RAF-1 subsequently phosphorylates MEK1 and MEK2 which activate respectively ERK1 and ERK2. The MAP kinases (MAPKs) are serine / threonine protein kinases e.g. ERK1/2 (extracellular signal-related kinase 1/2), which are activated by MAP/ERK kinases (MEKs), which in turn is activated by MEK kinase (MEKK), such as Raf. This pathway results in cell proliferation and in the increased transcription of Bcl2 family members and inhibitors of cell death proteins (IAPs), thereby promoting cell survival. Mitogenic sigalling increases the rate of translation of selective mRNAs [44]. Fig.1.7 shows the pathway of Ras

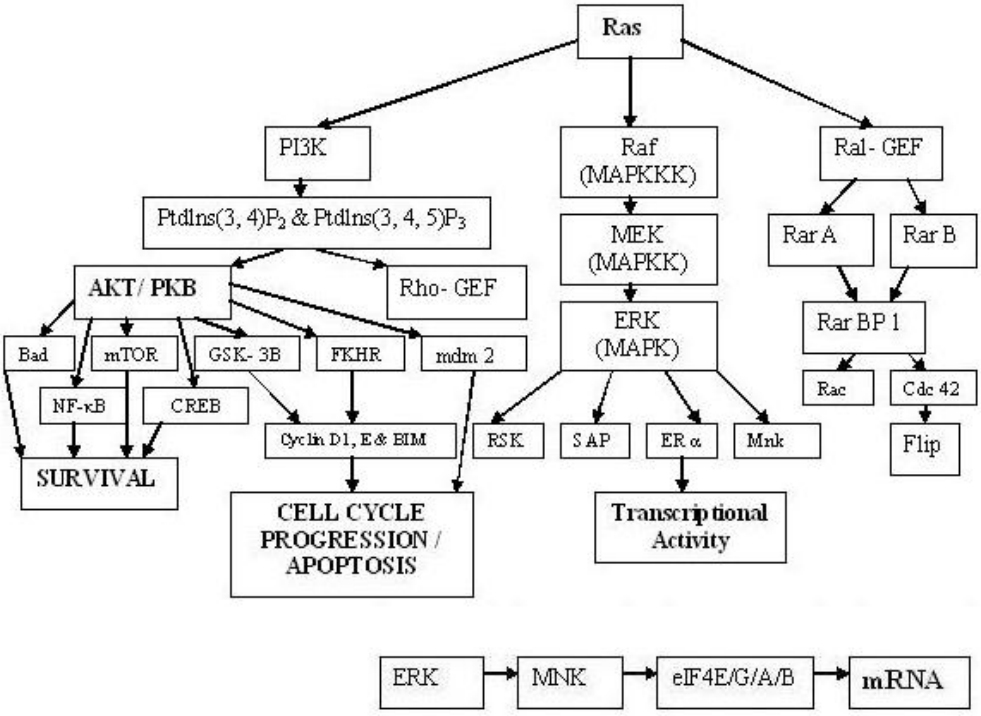


Fig. 1.7 Illustration of signal communication network triggered by RAS

- **JNK pathway :** The second most widely studied MAP kinase cascade is the JNK/SAPK (c-Jun NH_2-terminal kinase/stress activated protein kinase). The c-Jun kinase (JNK) is activated when cells are exposed to ultraviolet (UV) radiation, heat shock, or inflammatory cytokines. However, the functional consequence of JNK activation in UV-irradiated cells has not been established. The absence of JNK caused a defect in the mitochondrial death signaling pathway, including the failure to release cytochrome c.

- **p38 pathway:** The p38 kinase is the most well-characterized member of the MAP kinase family. It is activated in response to inflammatory cytokines, endotoxins, and osmotic stress. It shares about 50% homology with the ERKs. The upsteam steps in its activation of this cascade are not well defined. However, downstream activation of p38 occurs following its phosphorylation (at theTGY motif) by MKK3, a dual specificity kinase. Following its activation, p38 translocates to the nucleus and phosphorylates ATF-2. Another known target of p38 is MAPK2 that is involved in the phosphorylation and activation of heat-shock proteins. Although different MAP kinase cascades show high degree of specificity and functional separation, some degree of cross-talk is observed between different pathways shown in Fig 1.8. For example, JNKK, an activator of JNK/SAPK, is reported to activate p38, whereas MKK3 activates only p38 and not JNK/SAPK. MEKK1 that stimulates SEK/JNKK1 in the JNK/SAPK cascade has only a trivial effect on p38 activation. In the upstream signaling, SOS stimulates only the ERK pathways without affecting either JNK or p38 cascade. Another important observation is that if mammalian cells are treated with mitogenic agents; ERKs are significantly activated whereas JNK/SAPK is not affected. Conversely, cells exposed to stress cells activate JNK/SAPK pathway without altering the activity of ERKs. At the transcription level, ATF-2 is phosphorylated and activated by all three MAP kinases, whereas c-Jun and Elk-1 are phosphorylated by ERKs and JNK/SAPK, yet all these pathways result in transcriptional activity that is unique for a particular external stress.

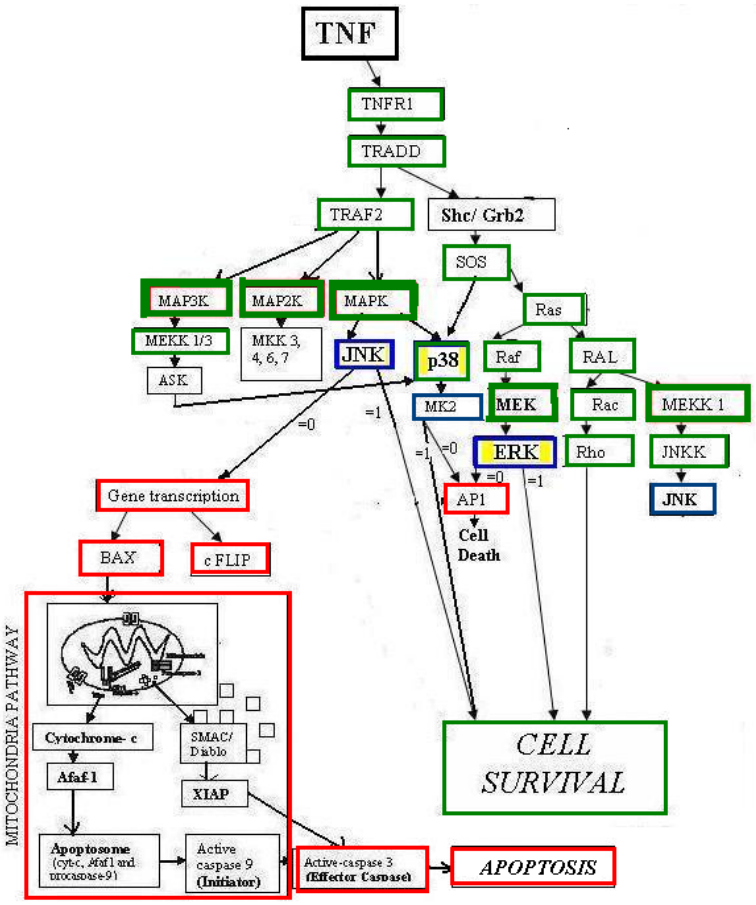


Fig. 1.8 Illustration of signal communication network triggered by MAP kinase.

1.3.3 INDUCTION OF DEATH SIGNALING: Like all death-domain containing members of the TNFR superfamily, TNF-R1 is involved in death signaling. However, TNF-induced cell death plays only a minor role compared to its overwhelming functions in the inflammatory process. Its death inducing capability is weak compared to other family members (such as Fas), and often masked by the anti-apoptotic effects of NF-κB. Nevertheless, TRADD binds FADD, which then recruits the cysteine protease caspase 8 shown in Figure 1.9. A high concentration of caspase 8 induces its autoproteolytic activation and subsequent cleaving of effector caspases, leading to cell cell death [11, 12].

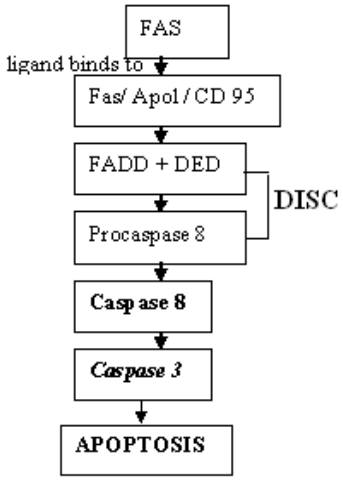


Fig. 1.9 Illustration of signal communication network triggered by FAS

Cell death is an essential strategy for the control of the dynamic balance in living systems, and two fundamentally different forms of cell death, ***cell death and necrosis***, have been defined. There are at least two broad pathways that lead to Cell death, an "*Extrinsic*" and an "*Intrinsic*" Pathway [10]. In both pathways, signaling results in the activation of a family of Cys (Cysteine) Proteases, named Caspases that act in a proteolytic cascade to dismantle and remove the dying cell.

In addition to their participation in survival and proliferation, PI3K and its target PKB/Akt, have emerged as critical signaling molecules that regulate multiple cellular processes. The ability of PI3K or Akt to suppress cell death has been attributed to both, Bad and caspase-9 phosphorylation, as well as ceramide regulation. In addition to these anti-apoptotic effects, Akt can also contribute activating NIK, with the consequent nuclear translocation of NF-κB. Thus, depending on cell context and cell type, TNF-α is able to induce cell survival or cell death pathways.

1.4 COMMUNICATION OF SIGNAL TRIGGERED BY EGF LEADING TO CELL SURVIVAL / CELL DEATH

The epidermal growth factor (EGF) and EGF receptor (EGFR) were among the first growth factor ligand-receptor pairs discovered [45]. Subsequently, EGFR was found to be a member of a receptor tyrosine kinase (RTK) family, the human epidermal growth factor receptor (HER) family. The epidermal growth factor receptor (EGFR) family plays

an important role in cell lineage determination, the morphogenesis of many organs and in cell survival in the adult. Moreover, activating mutants and over-expression of these family members contribute to oncogenesis by inducing cells to proliferate and to resist cell death. Upon ligand binding, EGFR dimerizes either with itself to form a homodimer or with other HER family members to form heterodimers (e.g., EGFR:HER2 or EGFR:HER3) [46, 47]. Ligand-induced dimerization causes a conformational change in the receptors that promotes the activation of the TK domain. Subsequent phosphorylation of the HER-kinase itself and/or other proteins, which then pass on to various signaling cascades [e.g., phosphoinositide 3-kinase (PI3K)/Akt and mitogen-activated protein kinase (MAPK) pathways], can lead to different cellular events such as growth, migration, and division

SIGNALING PATHWAY OF EGF

Upon ligand-binding receptors homo-dimerise or hetero-dimerise triggering tyrosine [48, 49] trans-phosphorylation of the receptor sub-units. These tyrosine phosphorylated sites allow proteins to bind through their Src homology 2 (SH2) domains leading to the activation of downstream signaling cascades including the RAS/extracellular signal regulated kinase (ERK) pathway, the phosphatidylinositol 3 kinase(PI3K) pathway and the activator of transcription (JAK/ STAT) pathway. Differences in the C-terminal domains of the ErbB receptors govern the exact second messenger cascades that are elicited conferring signaling specificity [50, 51]. The EGF signal is terminated primarily through endocytosis of the receptor-ligand complex. The contents of the endosomes are then either degraded or recycled to the cell surface [52, 53]. A number of signal transduction pathways branch out from the receptor signaling complex as shown in Fig. 1.10.

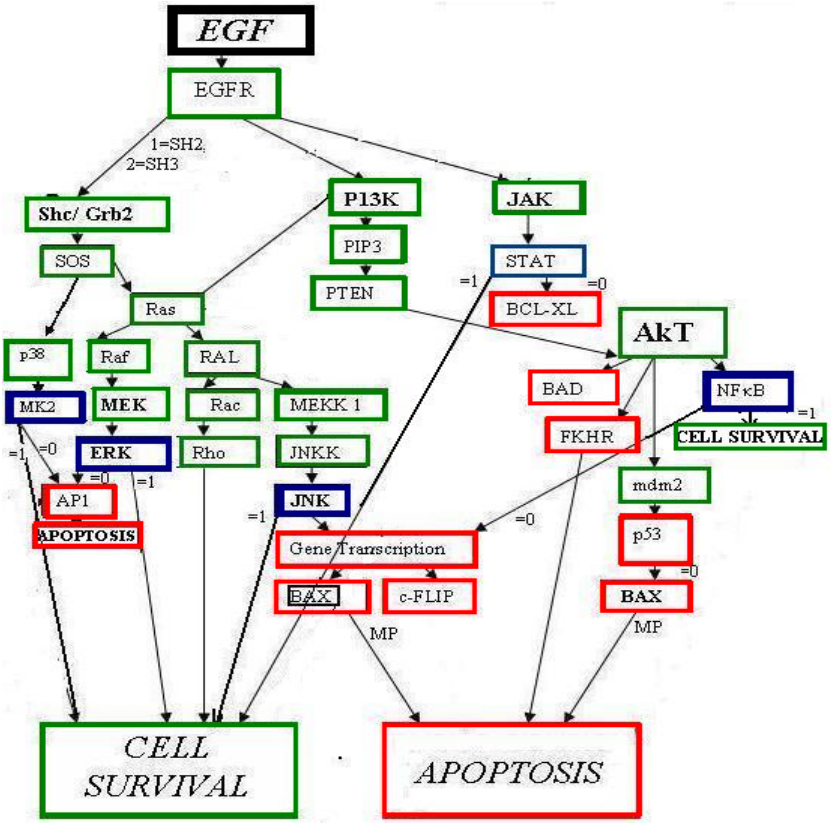


Fig. 1.10 Illustration of signal communication network triggered by EGF

1.4.1 ACTIVATION OF MAP KINASE PATHWAYS

MAP kinases are actually a family of protein kinases that are widely distributed and are found in all eukaryotic organisms. These can be classified into three main functional groups as shown in Fig. 1.11. The first is mediated by mitogenic and differentiation signals. The other two respond to stress and inflammatory cytokines. The ERK pathway responds to mitogen activation. In the JNK/SAPK pathway SAPK stands for stress activation protein kinase and within this class of kinases the Jun N-terminal kinases (JNK) for a subfamily. In the p38/HOG pathway HOG stands for high osmolarity glycerol where the p38 proteins are a subfamily. Each of these pathways led to the dual phosphorylation of MAP kinase family members responsible for activation of transcription factors.

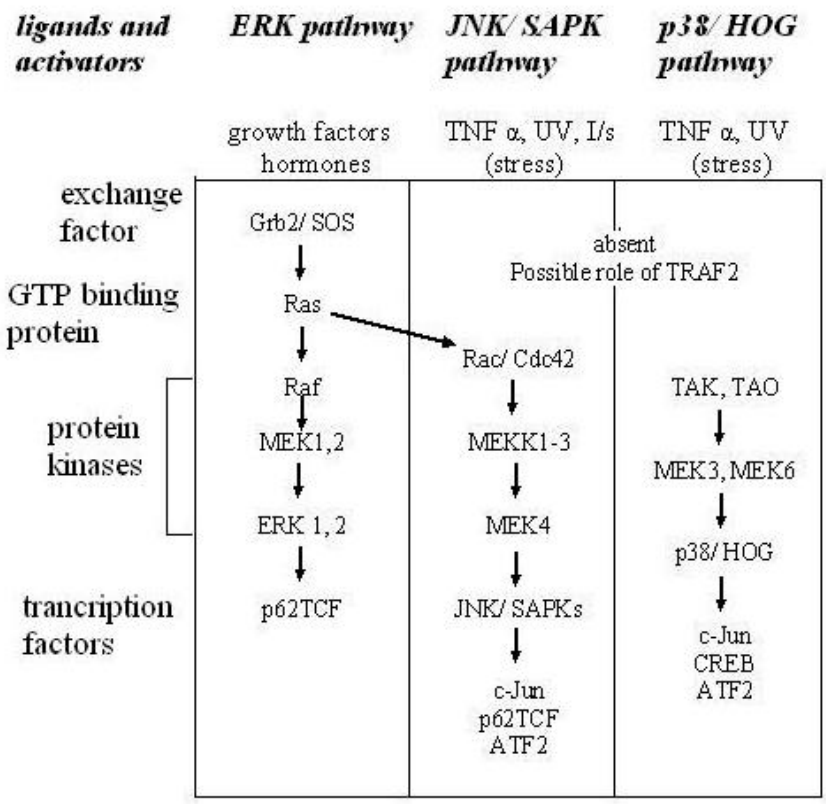


Fig. 1.11 Three main functional groups of MAP kinase

Cytokines and growth factors activate the mitogen-activated protein (MAP) kinase pathways resulting in the stimulation of ERK1/2, c-Jun N-terminal kinases and p38 kinases which in turn activate transcription factors like AP-1 and ATF-2. Other proinflammatory agents like TNF-α, IL-1 and LPS activate the transcription factor NF-κB which participates in the regulation of expression of immediate early genes involved in immune, acute phase and inflammatory responses. Besides, the transcription factors NF-κB and AP-1 which are immediate-early transcriptional activators and components of the JAK/STAT pathway play an important role in the transcriptional activation of many inflammatory genes. Consensus sequences for the transcription factors NF-κB, AP-1 and STAT1a have been found e.g. in the promoters of COX-2 and iNOS.

1.4.2 ACTIVATION OF PI3 kinase/AKT pathway

EGF also promotes cell survival through the activation of PI3 kinase/AkT signaling [54]. EGF triggers the recruitment of PI3 kinase to activated ErbB receptors, which is mediated by the binding of SH2 domains in PI3 kinase to phosphorylated tyrosine residues. The catalytic subunit of PI 3-kinase in turn phosphorylates phosphatidylinositol

(4, 5) biphosphate (PtdIns (4, 5)P_2) leading to the formation of PtdIns(3,4,5)P_3. PI 3-kinase can also activate RAS, resulting in the activation of ERK signaling, thereby facilitating cross-talk between survival pathways. A key downstream effector of PtdIns(3,4,5)P_3 is AKT. AKT promotes cell survival through the transcription of anti-apoptotic proteins. Intermediate transcription factors involved in this process are NFκB and CREB. Another downstream target of AKT is glucogen synthase kinase 3 (GSK3). Under basal conditions the constitutive activity of GSK3 leads to the phosphorylation and inhibition of a guanine nucleotide exchange factor eIF2B, which regulates the initiation of protein translation. Therefore, upon inactivation of GSK3 by AKT, eIF2B is dephosphorylated resulting in the promotion of protein synthesis and the storage of amino acids. AKT also activates mammalian target of rapamycin (mTOR), which promotes protein synthesis through p70 ribosomal S6 kinase (p70s6k) and inhibition of eIF-4E binding protein (4E-BP1) [55]. Collectively, these processes all promote cell growth and survival in response to EGF. Fig 1.12 which shows the pathway of PI3K.

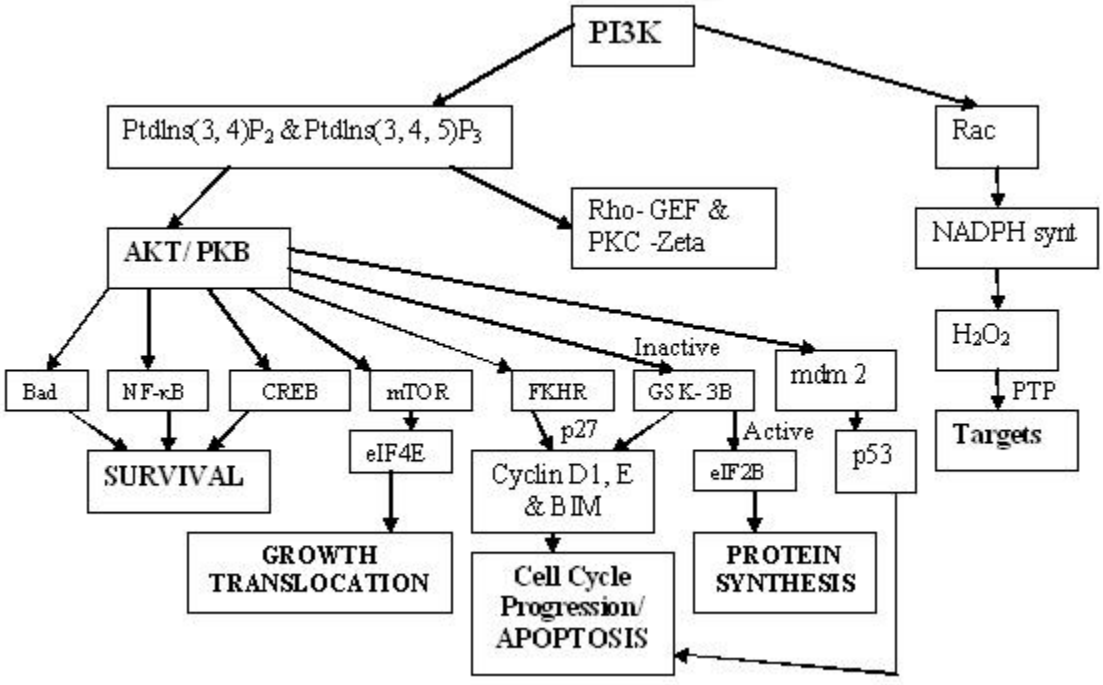


Fig. 1.12 Illustration of signal communication network triggered by PI3K

Following are the various proteins which helps in Cell survival/ Death using AkT .

1) $EGF / PI3K / AKT \rightarrow BAD\,(=1\,\text{Cell Survival}, =0\,\text{Cell Death});$ $(\text{Result Shown in Table 1.1})$

2) $EGF / PI3K / AKT \rightarrow FKHR\,(\text{Cell Death});$ $(\text{Result Shown in Table 1.2})$

3) $EGF / PI3K / AKT \rightarrow p53(=1\,\text{Cell Survival}, =0\,\text{Cell Death});$ $(\text{Result Shown in Table 1.3})$

5) $EGF / PI3K / AKT \rightarrow NF\kappa B\,(=1\,\text{Cell Survival}, =0\,\text{Cell Death});$ $(\text{Result Shown in Table 1.4})$

1. Bad : Bad was the first protein that is directly involved in cell death to be identified as a target of Akt. Bad is a member of the Bcl-2 family, which converges on the mitochondrial outer membrane to regulate cell. In the absence of Akt activity, Bad binds with another pro-survival member of the Bcl-2 family, Bcl-XL, and induces cell death, most likely by inhibiting the function of Bcl-XL to block the release of cytochrome *c* from mitochondria to the cytoplasm .However, activated Akt phosphorylates, Bad causing it to dissociate from Bcl-XL in the mitochondrial membrane and associate with the adaptor protein 14-3-3 instead. This results in the sequestration of Bad to the cytosol shown in Fig 1.13. Thus, Bad that is phosphorylated by Akt cannot induce cell death. Table 1.1 shows the truth table for cell death/ survival for AkT/ BAD pathway. In output '1' means survival and '0' means death.

The BAD protein is a pro-apoptotic member of the Bcl-2 family whose ability to hetero dimerize with survival proteins such as Bcl-X(L) and to promote cell death is inhibited by phosphorylation. JNK suppresses cell death in IL-3-depedent cells via phosphorylation of the pro-apoptotic Bcl-2 family protein BAD. In IL-3-dependent hematopoietic cells, IL-3 activated JNK phosphorylates BAD at Thr201. The phosphorylation results in reduced association of BAD with Bcl-xL, thereby suppressing cell death.

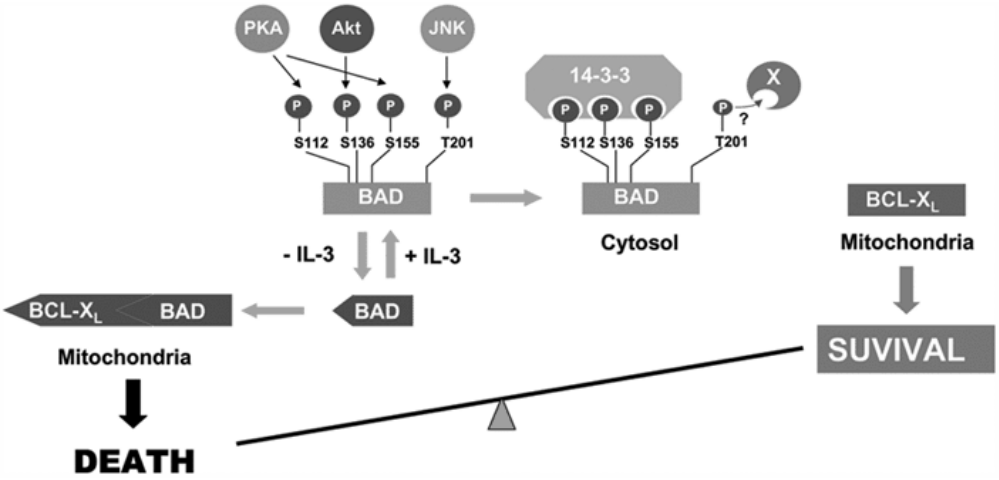


Fig. 1.13 Illustration of signal communication network triggered by BAD

Table 1.1 The truth table for cell death/ survival for AkT/ BAD pathway.

AkT	BAD	Output
0	0	0
0	1	0
1	0	0
1	1	1

2. Caspase 9 : During cell death, cytochrome c that is released into the cytoplasm binds the CED-4 homologue, Apaf-1. This causes it to bind, cleave, and activate the cysteine protease procaspase-9, which propagates the apoptotic caspase cascade that results in the activation of the 'executioner' caspases, caspase 3 and caspase 7. Interestingly, Akt phosphorylates procaspase-9 at Ser-196 rendering it resistant to processing and activation. Although it may appear redundant for Akt to act both upstream and downstream of cytochrome c in preventing cell death, the phosphorylation of procaspase-9 by Akt must have a physiological significance, as the cells that express caspase-9 with the Ser-196 mutated to alanine and underwent cell death that was resistant to Akt activity

3. FKHR1 : Akt phosphorylates and inactivates the Forkhead transcriptional factors. In the absence of survival signaling (i.e. phosphorylation by Akt), the Forkhead proteins enter the nucleus and are thought to induce the transcription of various cell-death related genes, such as FasL (Fas ligand) [56]. However, active Akt induces the phosphorylation of a specific site on the FKHR1 molecule that causes

it to be excluded from the nucleus, therefore losing its transcriptional activity. Table 1.2 shows the truth table for cell death/ survival for AkT/ FKHR pathway.

Table 1.2 The truth table for cell death/ survival for AkT/ FKHR pathway

AkT	FKHR	Output
0	0	0
0	1	0
1	0	1
1	1	0

4. **p53** : The mechanism by which p53 specifies the neuronal response to injury is poorly understood. However, currently available data suggest that the Bcl2 family member, Bax, is involved in p53-mediated neuronal death. Bax-deficient neurons are protected from cell death induced by DNA-damaging agents and adenovirus-mediated p53 over expression. Moreover, various forms of neuronal injury are associated with Bax translocation from the cytosol to the mitochondria. The redistribution of Bax to the mitochondria has been associated with a reduction in mitochondrial membrane potential, mitochondrial release of cytochrome c, and activation of caspases, suggesting that caspases may also be a component of a p53-induced cell death pathway. Recent studies indeed demonstrated that p53 is required for caspase activation in response to genotoxic stress. These findings suggest that some forms of neuronal injury invoke a common pathway involving signal transduction through p53, Bax, mitochondrial dysfunction, cytochrome c release and caspase activation.

   It is also critical that the pathways responsible for activating and suppressing p53 activity be identified. In this regard it is interesting to note that the important survival-promoting protein, Akt, can protect neurons from cell death by inhibiting p53-dependent transcriptional activity. These results demonstrate the interconnection that exists between pathways that govern cell death and viability and serve to remind us that the response and the outcome of neurons to stress are exceedingly complex. Table 1.3 shows the truth table for cell death/ survival for AkT/ p53 pathway.

Table 1.3 The truth table for cell death/ survival for AkT/ p53 pathway.

AkT	p53	Output
0	0	0
0	1	0
1	0	0
1	1	1

5. **NF-κB :** NF-κB is a factor that is involved in cell survival. It has been identified as a functional target of Akt. NF-κB is a family of transcription factors, which induce the expression of a wide variety of genes, especially those involved in survival, such as the Bcl-2 family member Bfl-1, and the caspase inhibitors c-IAP1 and c-IAP2. Binding with IκB sequesters it to the cytoplasm. Upon phosphorylation of IκB by IKKalpha and IKKbeta, IkB is degraded and NF-κB can enter the nucleus to induce transcription. It must be noted that NF-κB does not appear to be directly phosphorylated by Akt, but indirectly activated. Table 1.4 shows the truth table for cell death/ survival for AkT/ NF-κB pathway.

Table 1.4 The truth table for cell death/ survival for AkT/ NF-κB pathway.

AkT	NF-kB	Output
0	0	0
0	1	0
1	0	0
1	1	1

1.4.3 ACTIVATION OF JAK/STAT PATHWAY

Another signaling cascade initiated by EGF is the JAK/STAT pathway, which is also implicated in cell survival responses [57]. JAK phosphorylates STAT proteins are localized at the plasma membrane. This leads to the translocation of STAT proteins to the nucleus where they activate the transcription of genes associated with cell survival.

1.5 COMMUNICATION OF SIGNAL TRIGGERED BY INSULIN LEADING TO CELL SURVIVAL / CELL DEATH

Insulin is a hormone [58, 59] that regulates the amount of glucose (sugar) in the blood and is required for the body to function normally. It is synthesized as one chain, which is then cleaved at one site by a protease to form a two chain (A and B) protein. The two

chains are covalently linked by a bond (disulfide) between a cysteine, an amino acid with a $-CH_2SH$ side chain, with a cysteine side chain on the other chain. This protein binds to its receptor the insulin receptor, on cell membranes, which initiates a process of signal transduction [60]. The insulin receptor is a hormone-dependent kinase. When insulin binds on the extracellular part of the insulin receptor, shape changes communicated to the intracellular part cause it to bind ATP and phosphorylate proteins, specifically on tyrosine side chains. The insulin receptor is an insulin-dependent tyrosine kinase. Insulin binds to the extracellular α-subunit of the receptor and induces a conformational change that brings the α-subunits closer together. This leads to a rapid autophosphorylation of the receptor [61]. This then allows other intracellular proteins to bind to the intracellular domain of the receptor, and become phosphorylated. People who do not produce the necessary amount of insulin have diabetes. There are two general types of diabetes
The signaling pathway for insulin is shown in Fig. 1.14.

MAPK Pathway (Mitogenic responses) :

Other signal transduction proteins interact with IRS including GRB2, an adaptor protein that contains SH3 domains, which in turn associates with the guanine nucleotide exchange factor son-of sevenless (sos) and elicits activation of the MAPK cascade leading to mitogenic responses. SHC is another substrate for the insulin receptor. Upon phosphorylation SHC associates with GRB2 and can therefore activate the MAPK pathway independently of IRS. MAP kinases are actually a family of protein kinases that are widely distributed and are found in all eukaryotic organisms [62].

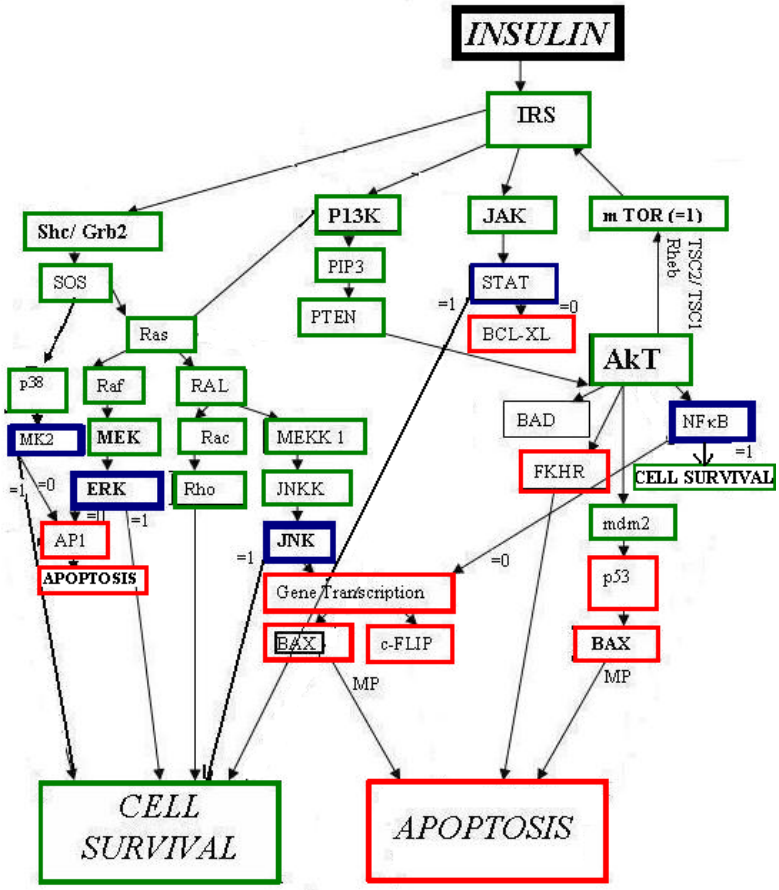


Fig. 1.14 Illustration of signal communication network triggered by Insulin

PI3K Pathway/ AkT: PI3K is activated by insulin, insulin-like growth factor-1 and other growth factors. PI3K is a heterodimeric lipid kinase with a broad range of cellular functions, including growth and differentiation, synthesis and degradation of carbohydrates, proteins and lipids, and membrane trafficking. PI3K consists of a regulatory subunit that associates with a catalytic subunit. The regulatory subunit binds the IRSs, whereas the catalytic subunit phosphorylates phosphatidylinositols in the membrane. PI3K is supposed to phosphorylate phosphatidylinositol 4, 5-bisphosphate at position 3 of the inositol ring to generate the putative lipid second messenger, phosphatidylinositol 3, 4, 5-trisphosphate [55]. Inhibition of PI3K by either Wortmannin or LY-294002 blocks the formation of phosphatidylinositol 3,4,5-trisphosphate within the cell and leads to the inhibition of several intracellular events, most importantly Glucose transporter 4 (GLUT4) translocation, thereby inhibiting insulin-stimulated glucose transport in the skeletal muscle. Activation of PI3K with insulin is insufficient for

insulin-stimulated glucose transport. A new pathway suggests that CAP (Cbl-associated protein)/Cbl may play a role in glucose uptake. CAP/Cbl is recruited to the insulin receptor in 3T3-L1 adipocytes and disruption of this interaction attenuates insulin-stimulated glucose transport. The importance of this pathway is unknown in human insulin-sensitive tissues and the role of this pathway in the skeletal muscle is not clear. The pathway for PI3K is shown in Fig. 4.5. PDK (phosphoinositide-dependent protein kinase)/Akt (protein kinase B, c-Akt) is one of the serine/threonine kinases downstream of PI3K. Akt was originally implicated in cancer development, promoting cell proliferation and inhibition of cell death. Insulin and other growth factors acutely activate Akt. Three isoforms of Akt have been identified: Akt1, Akt2 and Akt3, all of which are ubiquitously expressed. Full activation of Akt1 requires phosphorylation of two specific sites, Thr^{308} and Ser^{473} .Akt1 is activated by phosphorylation of Thr^{308} by PDK-1. The mechanism for phosphorylation of Ser^{473} is unclear, but PDK-2 is believed to be involved. Both Akt1 and Akt2 are involved in insulin signal transduction in skeletal muscle and adipose tissue. In contrast, Akt3 is not activated by insulin in the liver, muscle or adipose tissue. Whereas IRS-1 and PI3K phosphorylation/activation is impaired under *in vivo* and *in vitro* insulin stimulation in the skeletal muscle from Type II diabetic subjects, Akt phosphorylation is impaired only under *in vitro* conditions. Recently, a new Akt substrate, AS160, was identified in adipocytes. Initial studies demonstrate that insulin-stimulated AS160 phosphorylation is required for GLUT4 translocation leading to glucose transport.

Following are the various proteins which help in Cell survival/ Death using AkT .

1) $Insulin / PI3K / AKT \rightarrow BAD\,(=1\,\text{Cell Survival}, =0\,\text{Cell Death});$ (Result Shown in Table 1.1)

2) $Insulin / PI3K / AKT \rightarrow FKHR\,(\text{Cell Death});$ (Result Shown in Table 1.2)

3) $Insulin / PI3K / AKT \rightarrow p53(=1\,\text{Cell Survival}, =0\,\text{Cell Death});$ (Result Shown in Table 1.3)

4) $Insulin / PI3K / AKT \rightarrow NF\kappa B\,(=1\,\text{Cell Survival}, =0\,\text{Cell Death});$ (Result Shown in Table 1.4)

5) $Insulin / AKT / mTOR \rightarrow IRS\,(=1\,\text{Cell Survival}, =0\,\text{Cell Death});$ (Result Shown in Table 1.5)

mTOR : Using cell biological, biochemical, genomic and proteomic approaches, we are uncovering the complex molecular understanding of a signaling network centered around a G protein switch involving the tuberous sclerosis complex (TSC) tumor suppressors

(TSC1 and TSC2) and the Ras-related small G protein Rheb. A complex between TSC1 and TSC2 is regulated by multi-site phosphorylation and acts as a point of integration for a diverse array of cellular signals, including those arising from growth factors, nutrients and a variety of stress conditions. When active, the TSC1-TSC2 complex [55] acts as a GTPase activating protein (GAP) for Rheb, thereby turning Rheb off by stimulating its intrinsic GTPase activity. In the presence of growth factors and nutrients, this complex is turned off, allowing the GTP-bound active version of Rheb to accumulate and turn on downstream pathways. The best-characterized downstream effectors of Rheb is the mammalian target of rapamycin complex 1 (mTORC1), a critical regulator of cell growth and proliferation.

Table 1.5 The truth table for cell death/ survival for AkT/ mTOR pathway.

AkT	mTOR	Output
0	0	0
0	1	0
1	0	0
1	1	1

1.6 OBJECTIVE OF THE THESIS WORK

In this work our purpose is to determine whether the computational techniques such as electronic implementation (e.g.VHDL, CMOS and BICMOS, Fuzzy Logic) and mathematical modeling (e.g. Non–linear (ANN) and Deterministic implementation) can be used to uncover important aspects of biological cue-signal-response systems. Specifically we examine the tumor necrosis factor-alpha (TNF-α), epidermal growth factor (EGF) and insulin mediated cell survival/ cell death response of HT-29 human colon carcinoma cells based on heterogeneous measurements of nearly 10 signaling protein levels, states and activities. We wish to attract the interest of experimental molecular cell biologists with the strength of aforesaid computational techniques to find out the key players involve in biological processes before going for any experimental manipulations. A noteworthy feature of biological measurements reflects tangible molecular entities with known mechanistic roles in intracellular processes. Thus, these data-driven models of signaling are empirical, but not phenomenological, and suggest mechanistic dependencies. We showed here that the aforesaid methods can uncover key

contributors to death-survival decisions. These contributions always involve multiple proteins working in concert, but the informative proteins consist of only a fraction of the original protein dataset. Thus, our results suggest that within a cue-signal-response system lays a reduced set of information-rich protein measurements that together constitute an efficient model of the signaling network state and the relevant signal-response relationships.

This thesis focuses on system implementation of a signals and responses triggered by TNF-α, EGF and insulin factors leading to cell survival/ cell death. The computational techniques that have been used are: VHDL (Xilinx Tool), CMOS and BiCMOS (SPICE Tool), and Fuzzy Logic (MATLAB Tool), Non Linear Model (Artificial Neural Networks) and Deterministic Model taking three input signals. Each of these pieces of work has distinct characteristics. At the same time they are related to one another. To clearly and coherently demonstrate the goal, results and conclusion of each piece of work, we have arranged each work chapter wise in a publishing format. The format will benefit readers to understand the idea of development, conclusion, coherence and full significance as each chapter will be a full manuscript from background to conclusion at publication stage. In future we are also trying to develop bio-simulator that can be used for prediction of status of the cell leading to cell survival/ cell death giving chemotherapy and radiotherapy treatment.

1.7 APPROACH

Signal transduction pathways control cellular responses to stimuli, but it is unclear how molecular information is processed as a network. Large-scale collection and systematization of such data is likely to have a great impact on cell biology as complete genome sequencing has had on genetics. Cell signaling pathways interact with one another to form networks. Such networks are complex in their organization and exhibit emergent properties such as bistability and ultra sensitivity. Analysis of signaling networks requires a combination of experimental and theoretical approaches including the development and analysis of models [1, 2, 3]. This work examines signaling networks that control the survival decision treated with combinations of the pro-death cytokine,

tumor necrosis factor-α (TNF), and the pro-survival growth factors, ***epidermal growth factor*** (EGF) and ***insulin***.

The signaling system underlying cell death allows the cell to process input signals capturing information coming from the environment of the cell to lead to one of two possible outputs: cell survival or cell death. The system output is typically a phenotypic readout (death or survival); however, it can also be determined by measuring "early" signals that perfectly correlate with the death/ survival output. Examples of such early signals include phosphatidylserine exposure, membrane permeability, nuclear fragmentation and caspase substrate cleavage. The Fig. 1.15 illustrates the system under study linking the three input signaling such as TNF, EGF and Insulin and four output signals phosphatidylserine exposure, membrane permeability, nuclear fragmentation and caspase substrate cleavage leading to cell death/ survival.

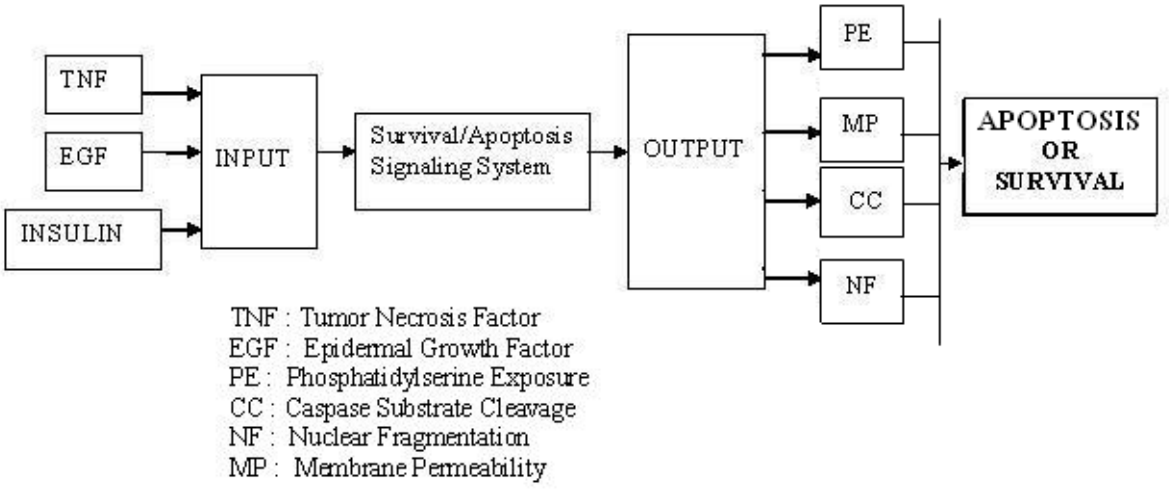


Fig. 1.15 Illustration of the system model of biological cue-signal-response system. In presence of three input signals (in single or in combination) the system model make logical decisions based on the prevailing intracellular and extra cellular conditions and produce a reliable behavior. In general the apoptotic cell death is described based on chromatin condensation, fragmentation of DNA, blebbing of the plasma membrane, and formation of "apoptotic bodies", which are phagocytes by neighboring cells.

Biological systems have the immense capability to generate complex structures from very simple systems. With simple rules and few inputs, a biological system can grow from a single cell to a multi cellular organism in a relatively short time. Biological systems, however, have been accurately synthesizing nano-scale machines for millions of years [63, 64, 65, 66]. Logic gates are the basic building blocks in electronic circuits that perform logical operations. These have input and output signals in the form of 0's and

1's; '0' signifies the absence of signal while '1' signifies its presence. Similar to the electronic logic gates, cellular components can serve as logic gates.

1.8 SUMMARY OF CONTRIBUTIONS:

In this dissertation, I make the following contributions:

1. **SPICE** : SPICE is a program that simulates electronic circuits in our PC. We can view any voltage or current waveform in our circuit. SPICE calculates these voltages and currents versus time (Transient Analysis) or versus frequency (AC Analysis). Most SPICE programs also perform other analysis like DC, Sensitivity, Noise and Distortion. Today, SPICE is available from many vendors who have added schematic drawing tools to the front end and graphics post processors to plot the results. SPICE simulators and applications have been expanded to analog and digital circuits, microwave devices, and electromechanical systems. Ideally, we would actually build and test actual circuits to understand all of its behavior. However, we would need breadboards, components and time to wire the circuit. Actual circuits also require expensive equipment like power supplies, signal generators and oscilloscopes. It may be difficult to physically breadboard every circuit one encounter. We can spend hours building an actual circuit and only get a simple concept from it, whereas, SPICE provides the insight in minutes. SPICE could be our "virtual" breadboard. Even if one has a short time to spare one can cover several circuit principles and applications.

2. **VHDL coding:** VHDL is a programming language that has been designed and optimized for describing the behavior of digital systems. It is a hardware description language that can be used to model a digital system at many levels of abstraction, ranging from the algorithmic level to the gate level. The complexity of the digital system being modeled could vary from that of simple gate to the complete digital electronic system or any thing in between. The digital system can also be described hierarchically. Timing can also be explicitly modeled in the same description. VHDL has many features appropriate for describing the behavior of electronic components ranging from simple logic gates to complete microprocessors and custom chips. Features of VHDL allow electrical aspects of circuit behavior (such as rise and fall times of signals, delays through gates, and functional operation) to be precisely

described. The resulting VHDL simulation models can then be used as building blocks in larger circuits (using schematics, block diagrams or system-level VHDL descriptions) for the purpose of simulation.

VHDL is also a general-purpose programming language; just as high-level programming languages allow complex design concepts to be expressed as computer programs, VHDL allows the behavior of complex electronic circuits to be captured into a design system for automatic circuit synthesis or for system simulation. Like Pascal, C and C++, VHDL includes features useful for structured design techniques, and offers a rich set of control and data representation features. Unlike these other programming languages, VHDL provides features allowing concurrent events to be described. This is important because the hardware described using VHDL is inherently concurrent in its operation. One of the most important applications of VHDL is to capture the performance specification for a circuit in the form of what is commonly referred to as a test bench. Test benches are VHDL descriptions of circuit stimuli and corresponding expected outputs that verify the behavior of a circuit over- time. Test benches should be an integral part of any VHDL project and should be created in tandem with other descriptions of the circuit.

3. **FUZZY LOGIC**: Fuzzy Logic is a departure from classical two-valued sets and logic that uses "soft" linguistic (e.g. large, hot, tall) system variables and a continuous range of truth values in the interval [0,1], rather than strict binary (True or False) decisions and assignments. Formally, fuzzy logic is a structured, model-free estimator that approximates a function through linguistic input/output associations. Fuzzy rule-based systems apply these methods to solve many types of "real-world" problems, especially where a system is difficult to model, is controlled by a human operator or expert, or where ambiguity or vagueness is common. A typical fuzzy system consists of a rule base, membership functions, and an inference procedure. Fuzzy logic is the part of artificial intelligence or machine learning which interprets a human’s actions. Computers can interpret only true or false values but a human being can reason the degree of truth or degree of falsehood. Fuzzy models interpret the human actions and are also called intelligent systems.

4. **Artificial Neural Network** : Artificial Neural Networks (ANN) are among the newest signal-processing technologies in the engineer's toolbox. The field is highly interdisciplinary but our approach will restrict to the view of the engineering perspective. In engineering, neural networks serve two important functions: as pattern classifiers and as nonlinear adaptive filters. We will provide a brief overview of the theory, learning rules, and applications of the most important neural network models. An Artificial Neural Network is an adaptive, most often nonlinear system that learns to perform a function (an input/output map) from data. Adaptive means that the system parameters change during operation, normally called the training phase. After the training phase the Artificial Neural Network parameters are fixed and the system is deployed to solve the problem at hand (the testing phase). The Artificial Neural Network is built with a systematic step-by-step procedure to optimize a performance criterion or to follow some implicit internal constraint, which is commonly referred to as the learning rule. The input/output training data are fundamental in Neural Network Technology, because they convey the necessary information to 'discover' the optimal operating point. The nonlinear nature of the neural network processing elements (PEs) provides the system with lots of flexibility to achieve practically any desired input/output map, i.e., some Artificial Neural Networks are universal mappers .

CHAPTER 2
ELECTRONICS IMPLEMENTATION

Bioelectronics encompasses a range of topics at the interface of biology and electronics. One aspect of the application of electronics in biology, medicine, and security includes both detection and characterization of biological materials, such as on the cellular and sub cellular level. Another aspect of bioelectronics is using biological systems in electronic applications (e.g., processing novel electronic components from DNA, nerves, or cells). Bioelectronics also focuses on physically interfacing electronic devices with biological systems (e.g., brain-machine, cell-electrode, or protein-electrode). Applications in this area include assistive technologies for individuals with brain-related disease or injury, such as paralysis, artificial retinas, and new technologies for protein structure-function measurements. Fig 2.1 shows the comparison of electronics and biological elements.

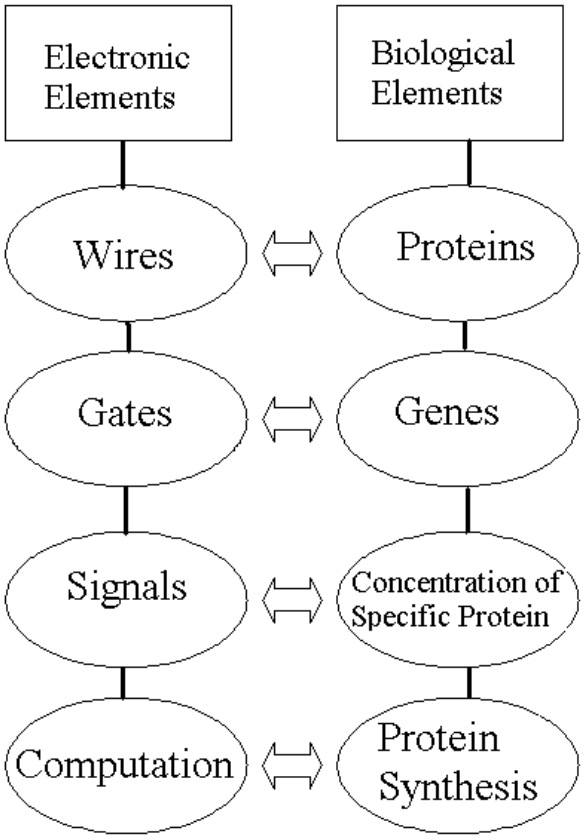


Fig 2.1 Comparison of Electronics and Biological Elements

2.1 SYSTEM MODEL OF CELL SURVIVAL/ CELL DEATH

The decision between cell survival/ cell death is well regulated by three input signals : TNF, EGF and insulin. These factors in single or in combination activate various key players in the network pertaining to cell survival/ cell death. Many proteins involved in this process that interact systematically regulating a specific pathway or cross talk with other proteins of different pathways. As a result many pathways activated simultaneously leading to many biochemical and physiological changes inside the cell. The final outcome of whether a cell dies or survives depends in the concentrations of key players among the pathways. In this work we have engineered regulatory network for cell death (Figure 2.2), and cell survival (Figure 2.3). Furthermore a combination model for both cell survival/ cell death was engineered shown in Figure 2.4.

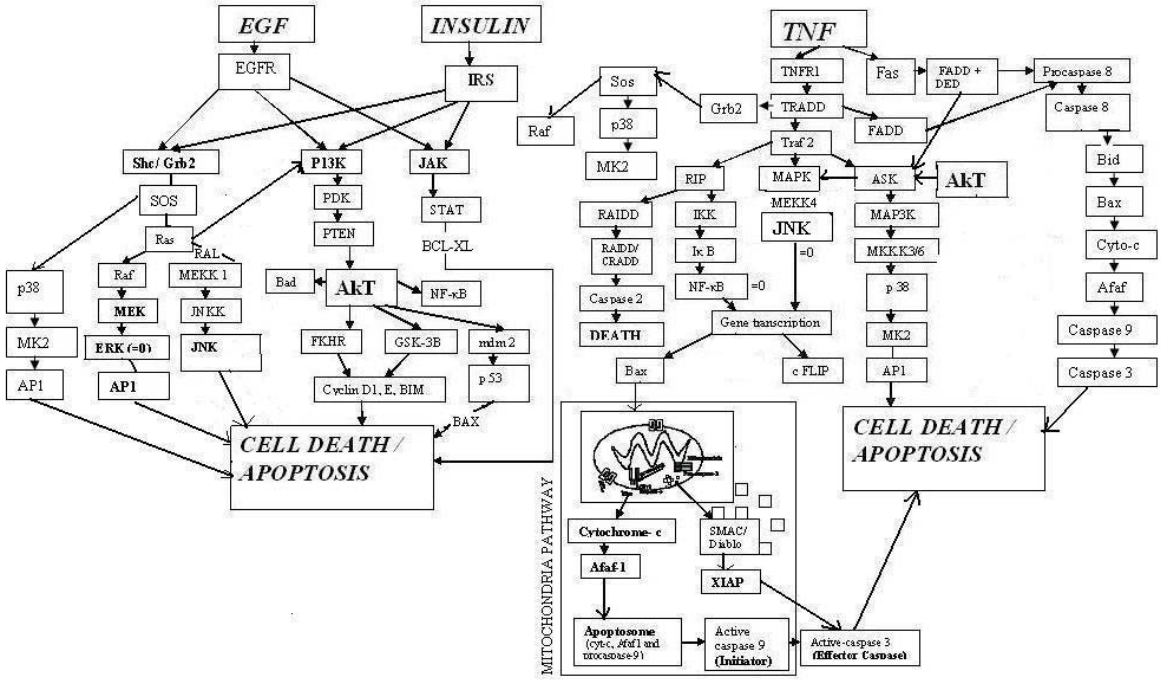


Fig 2.2 Illustration of cellular communication induced by combination of TNF, EGF and insulin leading to Cell death

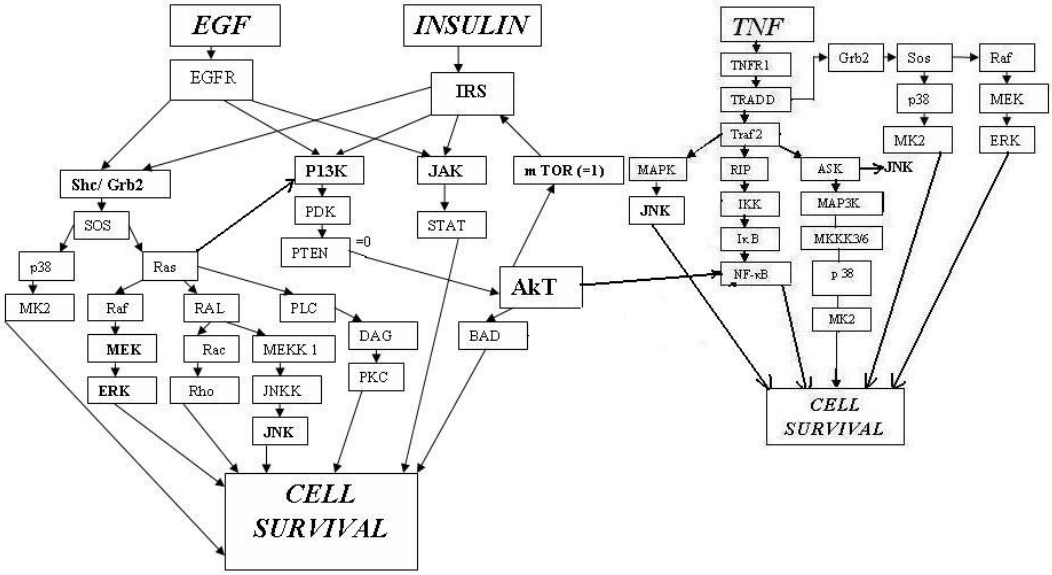


Fig 2.3 Illustration of cellular communication induced by combination of TNF, EGF and insulin leading to cell survival

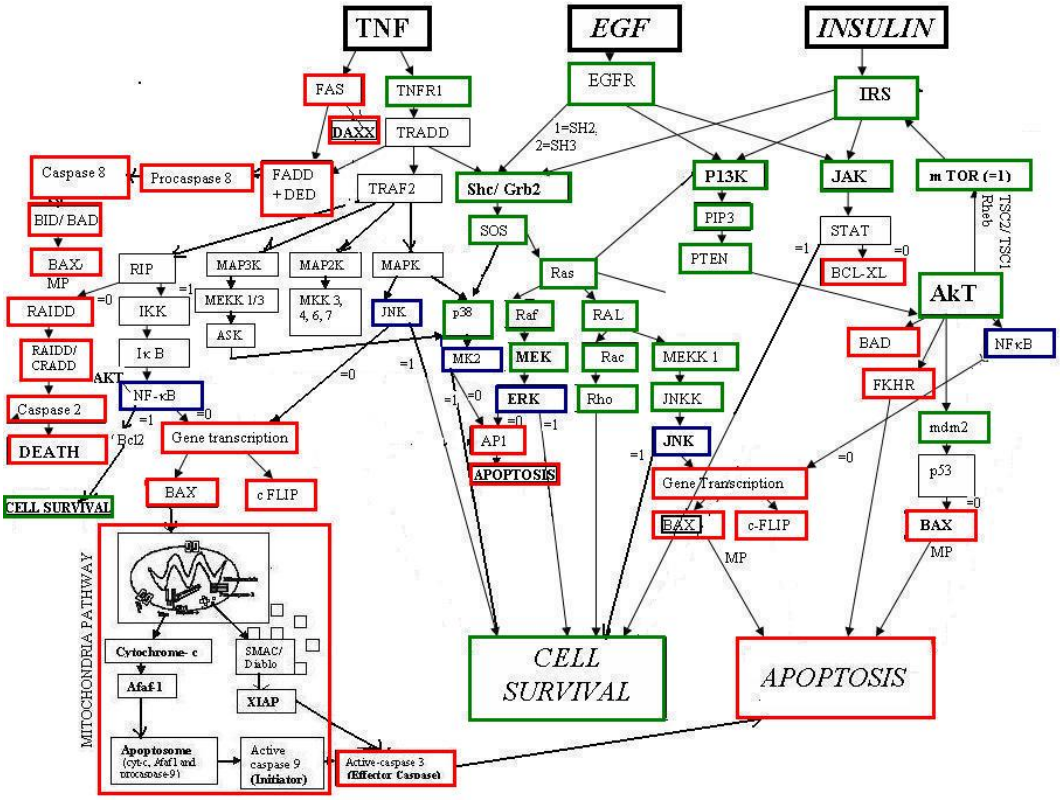


Fig 2.4 Illustration of cellular communication induced by combination of TNF, EGF and insulin leading to cell survival/ cell death

2.2 TRUTH TABLES

On the basis of above models we have made the *truth tables* for every possible pathways encountered for cell death/ survival for pro death cytokine *TNF*, and pro survival cytokine *EGF* and *Insulin* and combining the three taking '*1*' as *cell survival* and '*0*' as *cell death.* For cell survival the ten different proteins i.e. P13K, TNFR1, EGFR, IRS, IKK, Grb2, SOS, Ras, TRADD, Traf2 should be present. If any one of them is absent than there is a cell death. This is the necessary condition.

2.2.1 Truth Table for TNF

Raf	MEK	ERK	Output
0	0	0	0
0	0	1	0
0	1	0	0
0	1	1	0
1	0	0	0
1	0	1	0
1	1	0	0
1	1	1	1

RAL	MEKK	JNK	Output
0	0	0	0
0	0	1	0
0	1	0	0
0	1	1	0
1	0	0	0
1	0	1	0
1	1	0	0
1	1	1	1

RIP	IκB	NF-kB	Output
0	0	0	0
0	0	1	0
0	1	0	0
0	1	1	0
1	0	0	0
1	0	1	0
1	1	0	0
1	1	1	1

MAP3K	p 38	MK2	Output
0	0	0	0
0	0	1	0
0	1	0	0
0	1	1	0
1	0	0	0
1	0	1	0
1	1	0	0
1	1	1	1

FAS	Output
0	Survival
1	Death

2.2.2 Truth Table for EGF

RAL	RAC	Rho	Output
0	0	0	0
0	0	1	0
0	1	0	0
0	1	1	0
1	0	0	0
1	0	1	0
1	1	0	0
1	1	1	1

RAL	MEKK	JNK	Output
0	0	0	0
0	0	1	0
0	1	0	0
0	1	1	0
1	0	0	0
1	0	1	0
1	1	0	0
1	1	1	1

RAF	MEK	ERK	Output
0	0	0	0
0	0	1	0
0	1	0	0
0	1	1	0
1	0	0	0
1	0	1	0
1	1	0	0
1	1	1	1

PTEN	AkT	BAD	Output
0	0	0	0
0	0	1	0
0	1	0	0
0	1	1	1
1	0	0	0
1	0	1	0
1	1	0	0
1	1	1	0

PTEN	AkT	NFkB	Output
0	0	0	0
0	0	1	0
0	1	0	0
0	1	1	1
1	0	0	0
1	0	1	0
1	1	0	0
1	1	1	0

JAK	STAT	Output
0	0	0
0	1	0
1	0	0
1	1	1

p 38	MK2	Output
0	0	0
0	1	0
1	0	0
1	1	1

PTEN	AkT	p 53	Output
0	0	0	0
0	0	1	0
0	1	0	0
0	1	1	1
1	0	0	0
1	0	1	0
1	1	0	0
1	1	1	0

PTEN	AkT	FKHR	Output
0	0	0	0
0	0	1	0
0	1	0	1
0	1	1	0
1	0	0	0
1	0	1	0
1	1	0	0
1	1	1	0

2.2.3 Truth Table for Insulin

RAL	RAC	Rho	Output
0	0	0	0
0	0	1	0
0	1	0	0
0	1	1	0
1	0	0	0
1	0	1	0
1	1	0	0
1	1	1	1

RAL	MEKK	JNK	Output
0	0	0	0
0	0	1	0
0	1	0	0
0	1	1	0
1	0	0	0
1	0	1	0
1	1	0	0
1	1	1	1

RAF	MEK	ERK	Output
0	0	0	0
0	0	1	0
0	1	0	0
0	1	1	0
1	0	0	0
1	0	1	0
1	1	0	0
1	1	1	1

PTEN	AkT	BAD	Output
0	0	0	0
0	0	1	0
0	1	0	0
0	1	1	1
1	0	0	0
1	0	1	0
1	1	0	0
1	1	1	0

PTEN	AkT	NFkB	Output
0	0	0	0
0	0	1	0
0	1	0	0
0	1	1	1
1	0	0	0
1	0	1	0
1	1	0	0
1	1	1	0

PTEN	AkT	mTOR	Output
0	0	0	0
0	0	1	0
0	1	0	0
0	1	1	1
1	0	0	0
1	0	1	0
1	1	0	0
1	1	1	0

PTEN	AkT	p 53	Output
0	0	0	0
0	0	1	0
0	1	0	0
0	1	1	1
1	0	0	0
1	0	1	0
1	1	0	0
1	1	1	0

PTEN	AkT	FKHR	Output
0	0	0	0
0	0	1	0
0	1	0	1
0	1	1	0
1	0	0	0
1	0	1	0
1	1	0	0
1	1	1	0

p 38	MK2	Output
0	0	0
0	1	0
1	0	0
1	1	1

JAK	STAT	Output
0	0	0
0	1	0
1	0	0
1	1	1

2.2.4 Truth Table for TNF, EGF and Insulin combined

Raf	MEK	ERK	Output
0	0	0	0
0	0	1	0
0	1	0	0
0	1	1	0
1	0	0	0
1	0	1	0
1	1	0	0
1	1	1	1

RAL	MEKK	JNK	Output
0	0	0	0
0	0	1	0
0	1	0	0
0	1	1	0
1	0	0	0
1	0	1	0
1	1	0	0
1	1	1	1

mTOR	PTEN	AkT	Output
0	0	0	0
0	0	1	0
0	1	0	0
0	1	1	0
1	0	0	0
1	0	1	1
1	1	0	0
1	1	1	0

RIP	IκB	NF-κB	Output
0	0	0	0
0	0	1	0
0	1	0	0
0	1	1	0
1	0	0	0
1	0	1	0
1	1	0	0
1	1	1	1

p 38	MK2	Output
0	0	0
0	1	0
1	0	0
1	1	1

Above are all truth tables made from the computational model of Cell Survival and Cell death. In the later chapter we will implement all these truth tables using different electronic implementation.

2.3 EXPERIMENTAL FINDINGS OF MARKER PROTEINS LEADING TO CELL SURVIVAL/ CELL DEATH :

The experimental findings of cell survival or cell death with respect to cytokine treatments was taken from Gaudet *et al*. (2005); Janes *et al*. (2005) treated with ten cytokine combinations of tumor necrosis factor-α (TNF), a pro apoptotic cytokine, in combination

with epidermal growth factor (EGF) or insulin, two pro survival growth factors. The response of cell survival as well as cell death are regulated by eleven different proteins such as Mitogen-activated protein kinase-activated protein kinase 2 (MK2), c-jun N-terminal kinases(JNK), Forkhead transcription factor (FKHR), Mitogen-activated protein kinase and extracellular-regulated kinase kinase (MEK), Extracellular-regulated kinase (ERK), Insulin receptor substrate (IRS), AkT, IKK, Phospho-to-total EGFR (ptEGFR), Phospho-to-total Akt (ptAkt), pAkT. All the eleven proteins forms signaling network which leads to cell survival/ death. The response of signaling network is regulated by the concentration of cytokines like TNF, EGF and Insulin. Therefore, it is possible to built self consistent compendia cell signaling data based on the above eleven proteins that can be simulated computationally to yield important insites into the control of cell survival/ death. Kinases such as Akt and ERK were maximally active 5-15 min after cytokine addition whereas caspase cleavage was evident only after 4 hr time shown in Table 2.1. The changes in concentration of 11 marker proteins with respect to various treatments of cytokines are represented as heat map (Fig. 2.5 (*a*)). Similarly the output response in terms of (1) PS exposure (2) membrane permeability (3) DNA fragmentation and (4) caspases cleavage is represented as heat map in Fig 2.5(*b*).

Table 2.1 Combination of ten cytokine treatments used in the experimental study (Gaudet et al. 2005).

	(*a*)	(*b*)	(*c*)	(*d*)	(*e*)	(*f*)	(*g*)	(*h*)	(*i*)	(*j*)
TNF(ng/ml)	-	5	100	-	5	100	-	0.2	5	100
EGF(ng/ml)	-	-	-	100	1	100	-	-	-	-
Insulin(ng/ml)	-	-	-	-	-	-	500	1	5	500

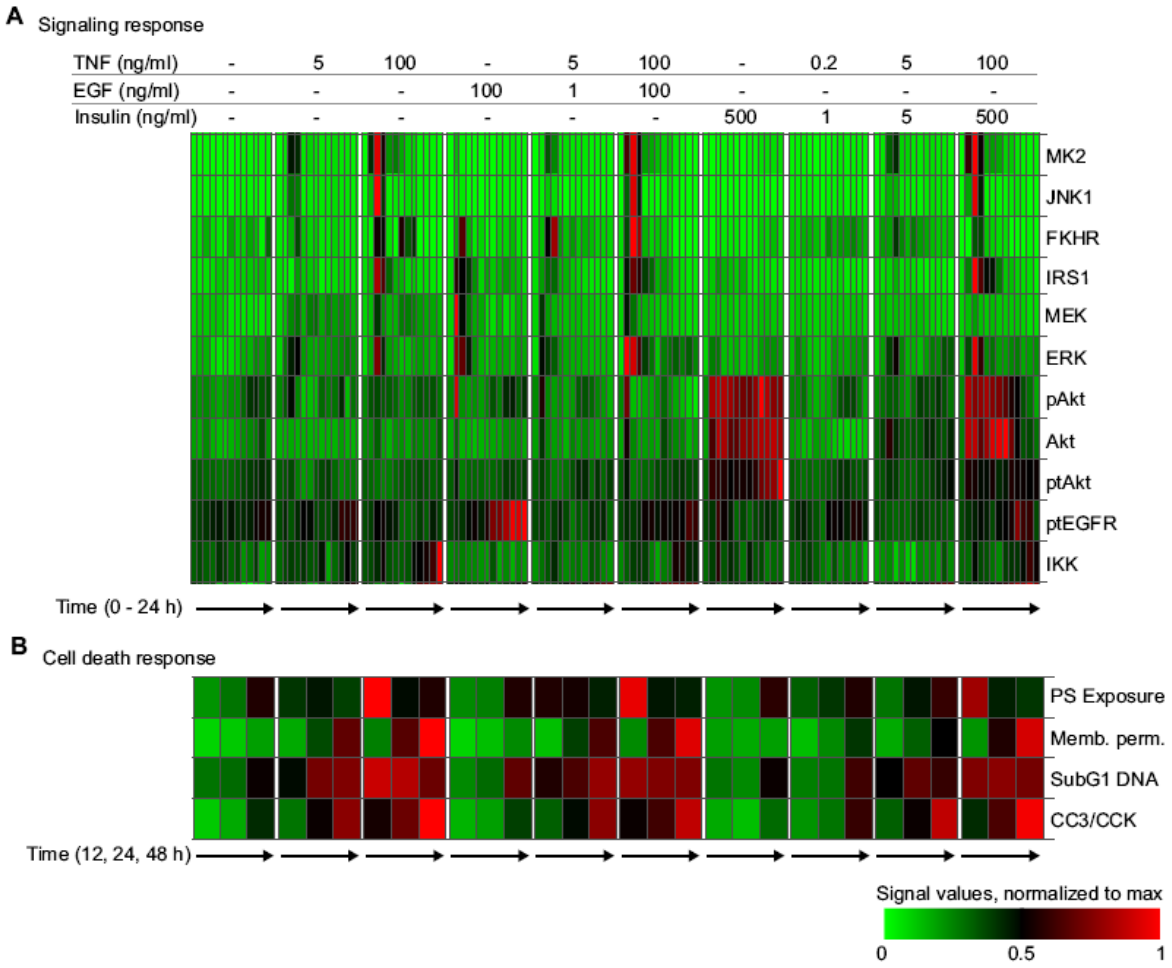


Fig 2.5 Heat map showing the level of the 11 marker proteins and the cell death response with respect to the treatments of TNF, EGF and Insulin with 10 cytokine combinations from Gaudet *et al* 2005 For each treatment, the average signal intensities were normalized to the maximal value obtained for that signal (0: green; 0.5: black; 1: red) and are plotted for the 13 time points . (**B**) Heat map of cell death responses for the 10 treatments. The average values from triplicate samples were normalized to the maximal value for that assay and are plotted for 12, 24, and 48 hr of treatment (0: green; 0.5: black; 1: red). The fractions of cells doubly positive for cleaved caspase-3 and cleaved cytokeratin (CC3/CCK) or with phosphatidylserine exposure (PS exposure), membrane permeability (Memb. Perm.), or sub-G1 DNA content (SubG1 DNA) or were measured by flow cytometry.

2.4 SYSTEM IMPLEMENTATION OF CMOS AND BICMOS USING SPICE

SPICE is a powerful general purpose analog and mixed-mode circuit simulator that is used to verify circuit designs and to predict the circuit behavior. This is of particular importance for integrated circuits. It was for this reason that SPICE was originally developed at the Electronics Research Laboratory of the University of California,

Berkeley (1975), as its name implies: **S**imulation **P**rogram for **I**ntegrated **C**ircuits **E**mphasis.

Today, SPICE is available from many vendors who have added schematic drawing tools to the front end and graphics post processors to plot the results. SPICE simulators and applications have expanded to analog and digital circuits, microwave devices, and electromechanical systems. Ideally, we would actually build and test actual circuits to understand all of its behavior. However, we would need breadboards, components and time to wire the circuit. Actual circuits also require expensive equipment like power supplies, signal generators and oscilloscopes. We can spend hours building an actual circuit and only get a simple concept from it, whereas, SPICE provides the insight in minutes. SPICE can be our "virtual" breadboard. Even if we have a short time to spare, we can cover several circuit principles and applications.

The Figure 2.6 summarizes the different steps involved in simulating a circuit with Capture and PSpice. We'll describe each of these briefly through a couple of examples.

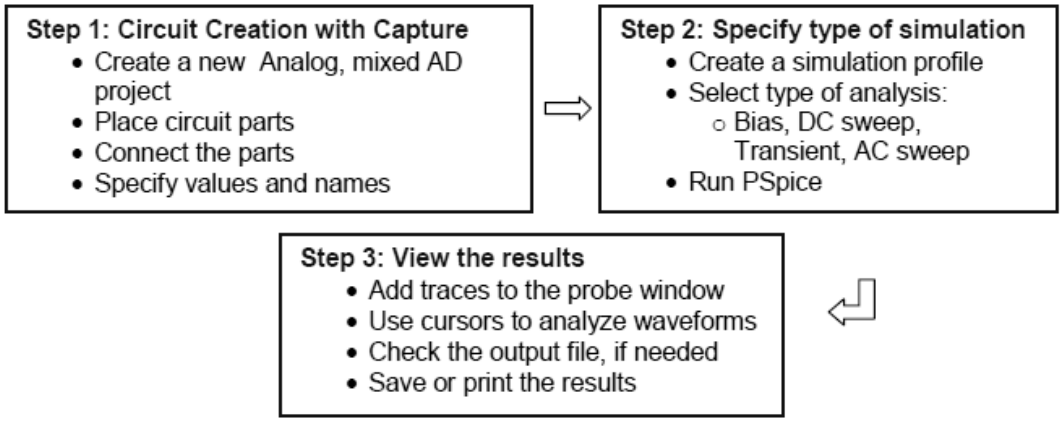


Fig 2.6 Steps involved in simulating a circuit using SPICE

The **metal–oxide–semiconductor field-effect transistor** (**MOSFET**, **MOS-FET**, or **MOS FET**) is a device used for amplifying or switching electronic signals. The basic principle of the device was first proposed by Julius Edgar Lilienfeld in 1925. In MOSFETs, a voltage on the oxide-insulated gate electrode can induce a conducting channel between the two other contacts called source and drain. The channel can be of *n*-type or *p*-type, and is accordingly called an *NMOSFET* or a *PMOSFET* (also commonly

*n*MOS, *p*MOS). It is by far the most common transistor in both digital and analog circuits, though the bipolar junction transistor was at one time much more common [67, 68].

A traditional metal–oxide–semiconductor (MOS) structure is obtained by growing a layer of silicon dioxide (SiO_2) on top of a silicon substrate and depositing a layer of metal or polycrystalline silicon (the latter is commonly used). As the silicon dioxide is a dielectric material, its structure is equivalent to a planar capacitor, with one of the electrodes replaced by a semiconductor.

A metal–oxide–semiconductor field-effect transistor (MOSFET) is based on the modulation of charge concentration by a MOS capacitance between a **body** electrode and a **gate** electrode located above the body and insulated from all other device regions by a gate dielectric layer which in the case of a MOSFET is an oxide, such as silicon dioxide. If dielectrics other than an oxide such as silicon dioxide (often referred to as oxide) are employed the device may be referred to as a metal–insulator–semiconductor FET. Compared to the MOS capacitor, the MOSFET includes two additional terminals (**source** and **drain**), each connected to individual highly doped regions that are separated by the body region. These regions can be either *p* or *n* type, but they must both be of the same type, and of opposite type to the body region. The source and drain (unlike the body) are highly doped as signified by a '+' sign after the type of doping [69, 70].

If the MOSFET is an n-channel or nMOS FET, then the source and drain are '*n*+' regions and the body is a '*p*' region. As described above, with sufficient gate voltage, above a threshold voltage value, electrons from the source (and possibly also the drain) enter the inversion layer or *n-channel* at the interface between the *p* region and the oxide. This conducting channel extends between the source and the drain, and current is conducted through it when a voltage is applied between source and drain. For gate voltages below the threshold value, the channel is lightly populated, and only a very small sub threshold voltage current can flow between the source and the drain.

If the MOSFET is a *p*-channel or *p*MOS FET, then the source and drain are '*p*+' regions and the body is a '*n*' region. When a negative gate-source voltage (positive

source-gate) is applied, it creates a *p-channel* at the surface of the *n* region, analogous to the *n*-channel case, but with opposite polarities of charges and voltages. When a voltage less negative than the threshold value (a negative voltage for *p*-channel) is applied between gate and source, the channel disappears and only a very small subthreshold current can flow between the source and the drain. Symbols for *n*MOS and *p*MOS is shown in Fig 2.7.

(*a*) (*b*)

Fig 2.7 Representation of (a) nMOS transistor (b) pMOS transistor

CMOS referred to as **complementary-symmetry metal–oxide–semiconductor** (or COS-MOS). The words "complementary-symmetry" refer to the fact that the typical digital design style with CMOS uses complementary and symmetrical pairs of *p*-type and *n*-typemetal oxide semiconductor field effect transistors (MOSFETs) for logic functions.

The principal reason for the success of the MOSFET was the development of digital CMOS logic, which uses *p*- and *n*-channel MOSFETs as building blocks. Overheating is a major concern in integrated circuits since ever more transistors are packed into ever smaller chips. CMOS logic reduces power consumption because no current flows (ideally), and thus no power is consumed, except when the inputs to logic gates are being switched. CMOS accomplishes this current reduction by complementing every *n*MOSFET with a *p*MOSFET and connecting both gates and both drains together [69, 70]. A high voltage on the gates will cause the *n*MOSFET to conduct and the *p*MOSFET not to conduct and a low voltage on the gates causes the reverse. During the switching time as the voltage goes from one state to another, both MOSFETs will conduct briefly. This arrangement greatly reduces power consumption and heat generation. Two important characteristics of CMOS devices are *high noise immunity* and *low static power consumption*. Significant power is only drawn while the transistors in the CMOS device are switching between on and off states. Consequently, CMOS devices do not produce as much waste heat as other forms of logic, for example transistor-transistor logic (TTL) or

NMOS logic, which uses all *n*-channel devices without *p*-channel devices. CMOS also allows a high density of logic functions on a chip.

Static CMOS Inverter

The composition of a *p*MOS transistor creates low resitance between its source and drain contacts when a low gate voltage is applied and high resistance when a high gate voltage is applied. On the other hand, the composition of an *n*MOS transistor creates high resistance between source and drain when a low gate voltage is applied and low resistance when a high gate voltage is applied.

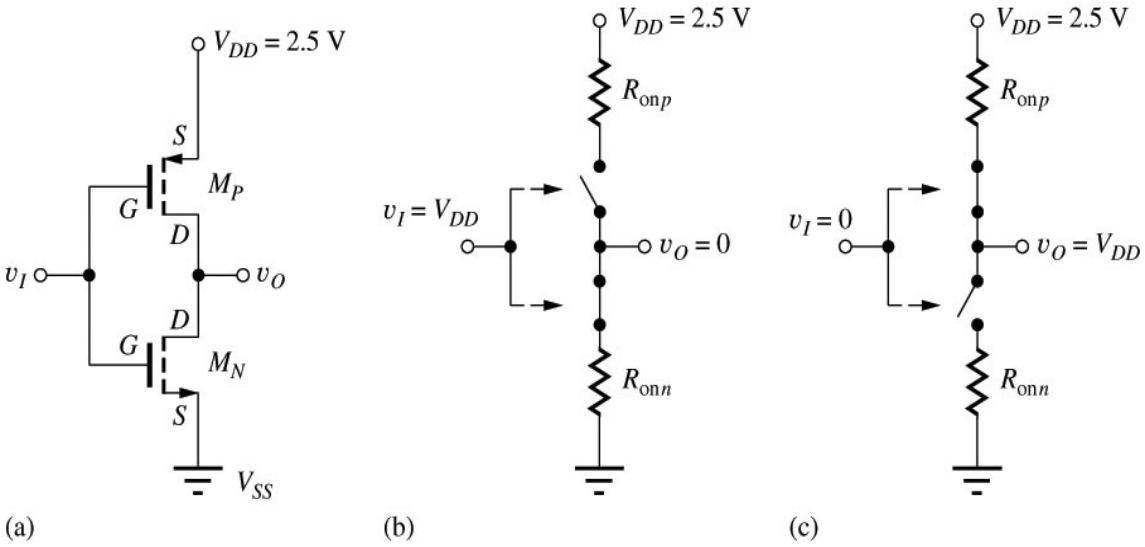


Fig 2.8 (a) Circuit schematic for a CMOS inverter (b) Simplified operation model with a high input applied (c) Simplified operation model with a low input applied

In Fig 2.8, when v_I is pulled high (to V_{DD}), the *p*MOS transistor is turned off, while the *n*MOS device is turned on pulling the output down to V_{SS}. When v_I is pulled low (to V_{SS}), the NMOS transistor is turned off, while the PMOS device is turned on pulling the output up to V_{DD}. In short, the outputs of the PMOS and NMOS transistors are complementary such that when the input is low, the output is high, and when the input is high, the output is low. Because of this opposite behavior of input and output, the CMOS circuits' output is the inversion of the input.

Duality

An important characteristic of a CMOS circuit is the duality that exists between its *p*MOS transistors and *n*MOS transistors. This can be easily accomplished by defining one in

terms of the NOT of the other. Due to the De Morgan's laws based logic, the PMOS transistors in parallel have corresponding NMOS transistors in series while the PMOS transistors in series have corresponding NMOS transistors in parallel.

a) CMOS NAND gate

More complex logic functions such as those involving AND and OR gates require manipulating the paths between gates to represent the logic. When a path consists of two transistors in *series*, then both transistors must have low resistance to the corresponding supply voltage, modeling an AND. When a path consists of two transistors in *parallel*, then either one or both of the transistors must have low resistance to connect the supply voltage to the output, modeling an OR.

Fig. 2.9 shows the circuit diagram of a NAND gate in CMOS logic. If both of the A and B inputs are high, then both the NMOS transistors (bottom half of the diagram) will conduct, neither of the PMOS transistors (top half) will conduct, and a conductive path will be established between the output and V_{ss} (ground), bringing the output low. If either of the A or B inputs is low, one of the NMOS transistors will not conduct, one of the PMOS transistors will, and a conductive path will be established between the output and V_{dd} (voltage source), bringing the output high.

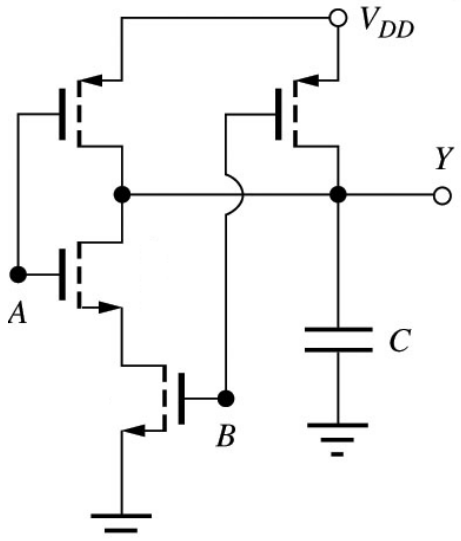


Fig 2.9 CMOS NAND gate implementation

An advantage of CMOS over NMOS is that *both* low-to-high and high-to-low output transitions are fast since the pull-up transistors have low resistance when switched on, unlike the load resistors in NMOS logic. In addition, the output signal swings the full voltage between the low and high rails. This strong, more nearly symmetric response also makes CMOS more resistant to noise.

b) CMOS NOR gate : Fig. 2.10 shows the circuit diagram of a NOR gate in CMOS logic.

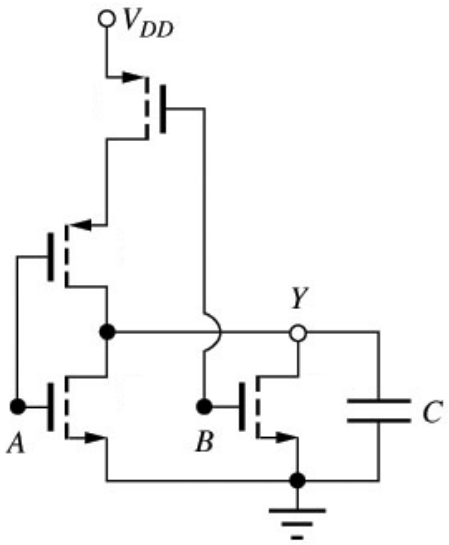


Fig 2.10 CMOS NOR gate implementation

SYSTEM IMPLEMENTATION USING BiCMOS

In integrated circuit technologies, **BiCMOS**, also called **BiMOS**, refers to the integration of bipolar junction transistors and CMOS technology into a single integrated circuit device. This technology has commercial application in amplifier and discrete component logic design.

Advantages of CMOS over bipolar

- Lower static power dissipation
- Higher noise margins
- Higher packing density – lower manufacturing cost per device
- High yield with large integrated complex functions

- High input impedance (low drive current)
- Scaleable threshold voltage
- High delay sensitivity to load (fan-out limitations)
- Low output drive current (issue when driving large capacitive loads)
- Low trans conductance, where trans conductance, $g_m \alpha V_{in}$
- Bi-directional capability (drain & source are interchangeable)
- A near ideal switching device

Advantages of Bipolar over CMOS

- Higher switching speed
- Higher current drive per unit area, higher gain
- Generally better noise performance and better high frequency characteristics
- Better analogue capability
- Improved I/O speed (particularly significant with the growing importance of package limitations in high speed systems).
- high power dissipation
- lower input impedance (high drive current)
- low voltage swing logic
- low packing density
- low delay sensitivity to load
- high trans conductance, where trans conductance, $g_m \alpha V_{in}$
- high unity gain band width at low currents
- essentially unidirectional

Combine advantages in BiCMOS Technology

- It follows BiCMOS technology that goes some way towards combining the virtues of both CMOS and Bipolar technologies

- Design uses CMOS gates along with bipolar totem-pole stage where driving of high capacitance loads is required

Resulting benefits of BiCMOS technology over solely CMOS or solely bipolar :

- Improved speed over purely-CMOS technology
- Lower power dissipation than purely-bipolar technology (simplifying packaging and board requirements)
- Flexible I/Os (i.e., TTL, CMOS or ECL) – BiCMOS technology is well suited for I/O intensive applications. ECL, TTL and CMOS input and output levels can easily be generated with no speed or tracking consequences
- high performance analogue

THE BiCMOS INVERTER : Fig 2.11 shows the circuit diagram of Bi CMOS inverter. Two bipolar transistors (T_3 *and* T_4), one *n*MOS and one *p*MOS transistor (both enhancement-type devices, OFF at $V_{in}=0V$). The MOS switches perform the logic function & bipolar transistors drive output loads

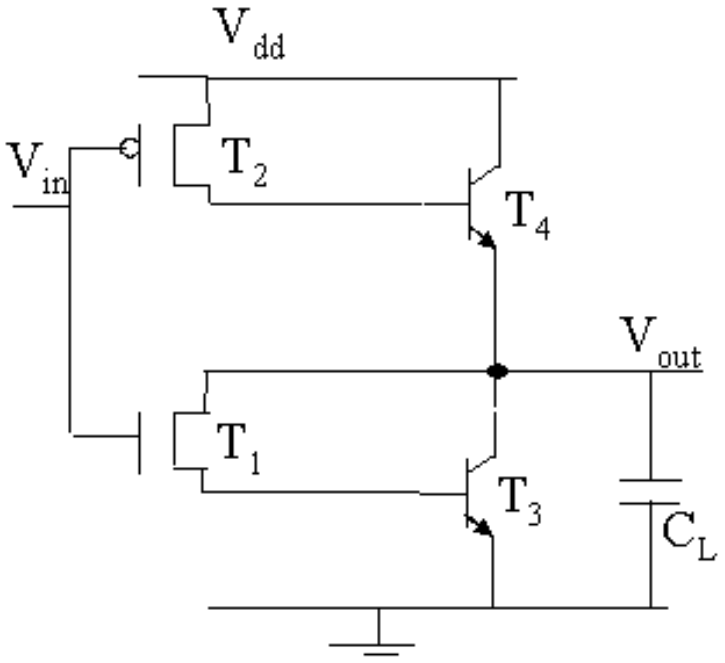


Fig 2.11 BiCMOS Inverter

When $\mathbf{V_{in}} = \mathbf{0}$: T_1 is off. Therefore T_3 is non-conducting, T_2 ON - supplies current to base of T_4. T_4 base voltage set to V_{dd}. T_4 conducts and acts as current source to charge load C_L towards V_{dd}. V_{out} rises to V_{dd} - V_{be} (of T_4)

Note : $V_{be\ (of\ T4)}$ *is base-emitter voltage of* T_4.

$$5V - V_{be} \text{ (of T4)}$$

When $\boldsymbol{V_{in}} = \boldsymbol{V_{dd}}$: T_2 is off. Therefore T_4 is non-conducting. T_1 is ON and supplies current to the base of T_3 . T_3 conducts and acts as a current sink to discharge load C_L towards 0V. V_{out} falls to $0V + V_{CEsat}$ (of T_3)

Note : $V_{CEsat\ (of\ T3)}$ *is saturation V from* T_3 *collector to emitter.*

BiCMOS NAND GATE

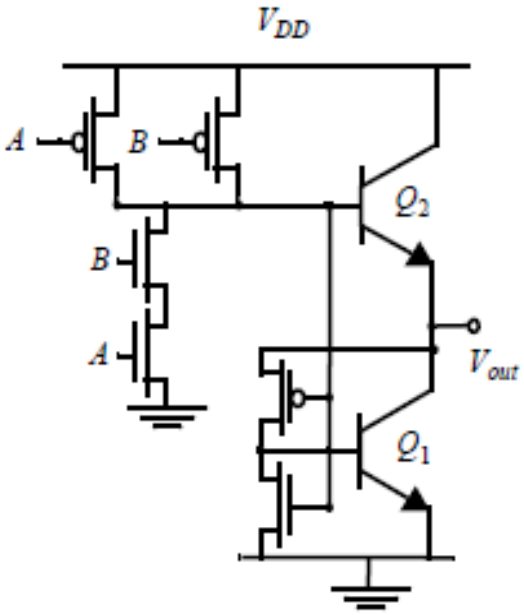


Fig 2.12 Two input BiCMOS NAND gate

ADVANTAGES AND DISADVANTAGES OF BiCMOS TECHNOLOGY :

Advantages of BiCMOS Technology :

- Analogue amplifier design is facilitated and improved
- High impedance CMOS transistors may be used for the input circuitry while the remaining stages and output drivers are realized using bipolar transistors

- In general, BiCMOS devices offer many advantages where high load current sinking and sourcing is required. The high current gain of the NPN transistor greatly improves the output drive capability of a conventional CMOS device.
- MOS speed depends on device parameters such as saturation current and capacitance. These in turn depend on oxide thickness, substrate doping and channel length.
- Compared to CMOS, BiCMOS's reduced dependence on capacitive load and the multiple circuit and I/Os configurations possible greatly enhance design flexibility and can lead to reduced cycle time (i.e., faster circuits).
- BiCMOS is inherently robust with respect to temperature and process variations, resulting in less variability in final electrical parameters, resulting in higher yield, an important economic consideration.
- Large circuits can impose severe performance penalties due to simultaneously switching noise, internal clock skews and high nodal capacitances in critical paths - BiCMOS has demonstrated superiority over CMOS in all of these factors.
- BiCMOS can take advantage of any advances in CMOS and/or bipolar technology, greatly accelerating the learning curve normally associated with new technologies.

Disadvantages with BiCMOS technology

- Main disadvantage : greater process complexity compared to CMOS
- Results in a 1.25 -> 1.4 times increase in die costs over conventional CMOS.
- Taking into account packaging costs, the total manufacturing costs of supplying a BiCMOS chip ranges from 1.1-> 1.3 times that of CMOS.
- However, as CMOS complexity has increased, the percentage difference between CMOS and BiCMOS mask steps has decreased.

2.4.1 RESULTS AND DISCUSSIONS : We had studied relating how TNF, EGF and Insulin work and its pathways in detail and explain each and every possible path for that. Based on pathways we had made truth tables for every possible path for cell

survival/cell death. Than we realize the truth tables by Karnaugh Map (K-Map) and get the Boolean expression for its individual possible paths. We simulate the results of each path, then combine all the results, and simulate through SPICE simulator using CMOS and BiCMOS, get result of TNF, EGF and Insulin for its cell survival/ cell death. In output, '1' signifies cell survival and '0' signifies cell death. For cell survival the ten different proteins i.e. P13K, TNFR1, EGFR, IRS, IKK, Grb2, SOS, Ras, TRADD, Traf2 should present. If any one of them is absent than there is a cell death.

2.4.1.1 TNF signal cascade : The response of input signal TNF, in presence of key players regulating different pathways by using CMOS is shown in Fig 2.13 and by using BiCMOS is shown in Fig 2.14. As the ten marker proteins are present ('1') then, it activates five different pathways. V(35) is represented as output for CMOS and V(49) is represented as output for BiCMOS. Details of input notation for CMOS and BiCMOS are shown in Table 2.2.

Table 2.2 : Different key proteins involved in communicating signal of TNF through different pathways for CMOS and BiCMOS implementation

	Input		**Combination of marker proteins**
	CMOS	**BiCMOS**	
Pathway 1	V(5)	V(8)	RAF, MEK, ERK
Pathway 2	V(11)	V(16)	RAL, MEKK, JNK
Pathway 3	V(7)	V(24)	RIP, IκB, NFκB
Pathway 4	V(23)	V(31)	MAP3k, p38, MK2
Pathway 5	V(34)	V(9)	FAS

Now to activate first pathway all the three marker proteins should be present ('1') than only it will lead to cell survival. Similarly for pathways 2, 3, and 4. But to activate pathway 5, marker protein FAS should be absent ('0') than it will lead to cell survival.

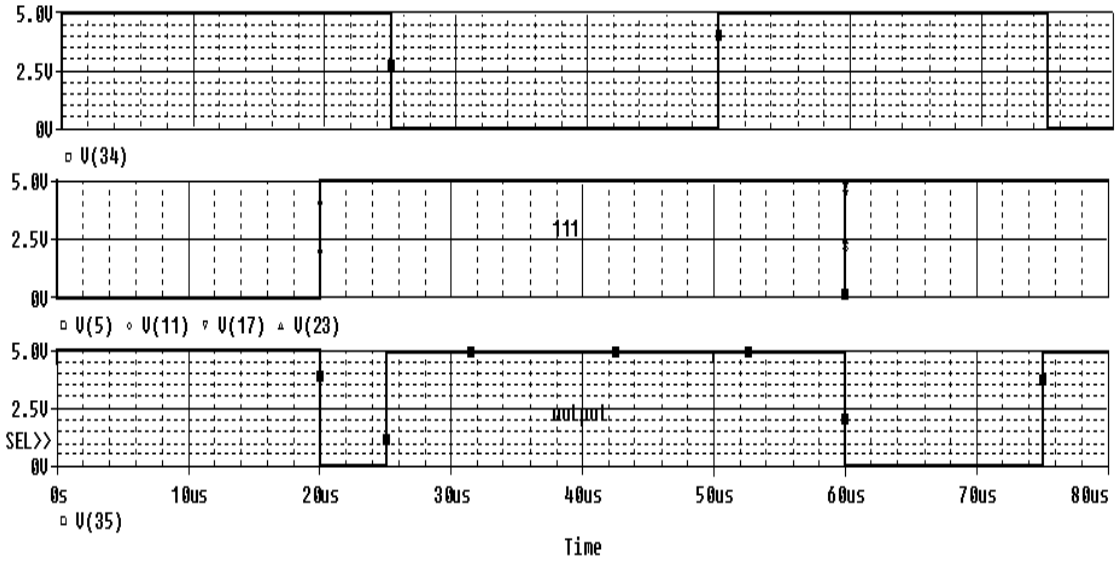


Fig 2.13 System Implementation of TNF signal cascade using CMOS

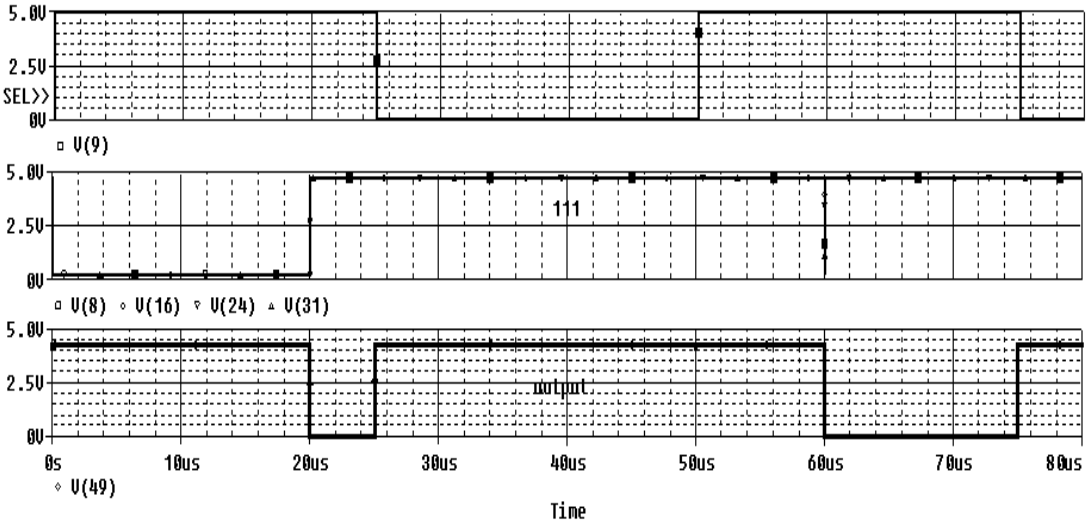


Fig 2.14 System Implementation of TNF signal cascade using BiCMOS

2.4.1.2 EGF signal cascade : The response of input signal EGF, in presence of key players regulating different pathways by using CMOS is shown in Fig 2.15 and by using BiCMOS is shown in Fig 2.16. As the ten marker proteins are present (‘1’) then, it activates five different pathways. V(70) is represented as output for CMOS and V(106) is represented as output for BiCMOS. Details of input notation for CMOS and BiCMOS are shown in Table 2.3.

Table 2.3 : Different key proteins involved in communicating signal of EGF through different pathways for CMOS and BiCMOS implementation

	Input		Combination of marker proteins
	CMOS	**BiCMOS**	
Pathway 1	V(5)	V(8)	RAL, RAC, Rho
Pathway 2	V(11)	V(16)	RAL, MEKK, JNK
Pathway 3	V(17)	V(24)	RAF, MEK, ERK
Pathway 4	V(28)	V(36)	p38, MK2
Pathway 5	V(44)	V(61)	PTEN, AkT, NFκB
Pathway 6	V(51)	V(71)	PTEN, AkT, BAD
Pathway 7	V(58)	V(81)	PTEN, AkT, p53
Pathway 8	V(67)	V(94)	PTEN, AkT, FKHR
Pathway 9	V(32)	V(41)	JAK, STAT

Now to activate first pathway, all the three marker proteins should be present (‘1’) than only it will lead to cell survival. Similarly for pathways 2, 3, 4 and 9 all the marker proteins should present. To activate pathway 5, 6 and 7 marker protein PTEN should be absent (‘0’) and to activate pathway 8 PTEN and FKHR should be absent (‘0’) than it will lead to cell survival.

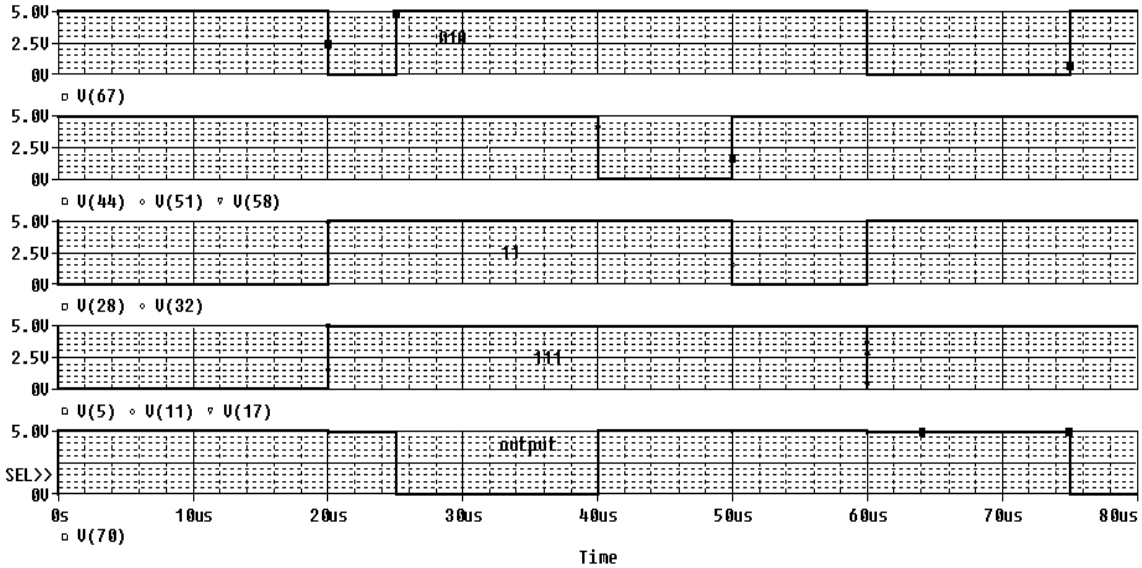


Fig 2.15 System Implementation of EGF signal cascade using CMOS

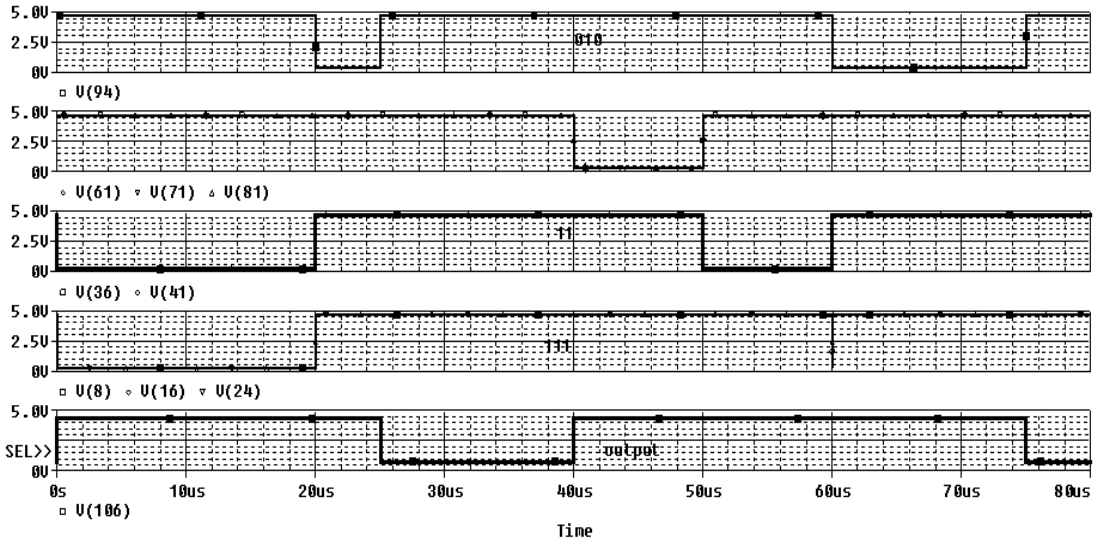


Fig 2.16 System Implementation of EGF signal cascade using BiCMOS

2.4.1.3 Insulin signal cascade : The response of input signal Insulin, in presence of key players regulating different pathways by using CMOS is shown in Fig 2.17 and by using BiCMOS is shown in Fig 2.18. As the ten marker proteins are present ('1') then, it activates five different pathways. V(81) is represented as output for CMOS and V(124) is represented as output for BiCMOS. Details of input notation for CMOS and BiCMOS are shown in Table 2.4.

Table 2.4 : Different key proteins involved in communicating signal of Insulin through different pathways for CMOS and BiCMOS implementation

	Input		**Combination of marker proteins**
	CMOS	**BiCMOS**	
Pathway 1	V(5)	V(8)	RAL, RAC, Rho
Pathway 2	V(11)	V(16)	RAL, MEKK, JNK
Pathway 3	V(17)	V(24)	RAF, MEK, ERK
Pathway 4	V(28)	V(36)	p38, MK2
Pathway 5	V(44)	V(61)	PTEN, AkT, NFκB
Pathway 6	V(51)	V(71)	PTEN, AkT, BAD
Pathway 7	V(58)	V(81)	PTEN, AkT, p53
Pathway 8	V(67)	V(94)	PTEN, AkT, FKHR
Pathway 9	V(37)	V(51)	PTEN, AkT, mTOR
Pathway 10	V(32)	V(41)	JAK, STAT

Now to activate first pathway, all the three marker proteins should be present ('1') than only it will lead to cell survival. Similarly for pathways 2, 3, 4, and 10 all the marker proteins should present. To activate pathway 5, 6 7 and 9 marker protein PTEN should be

absent (‘0’) and to activate pathway 8, PTEN and FKHR should be absent (‘0’) than it will lead to cell survival.

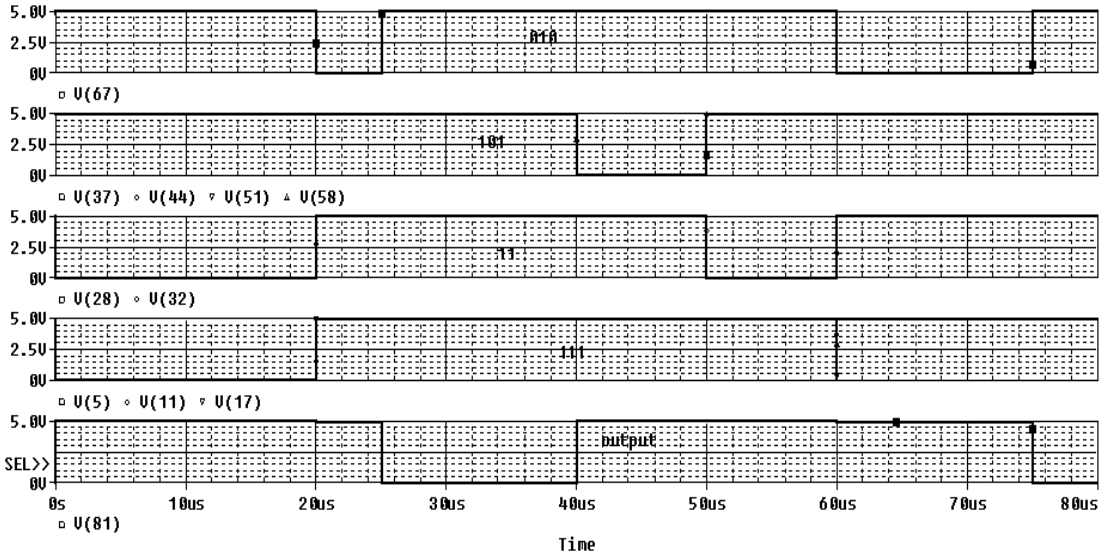


Fig 2.17 System Implementation of Insulin signal cascade using CMOS

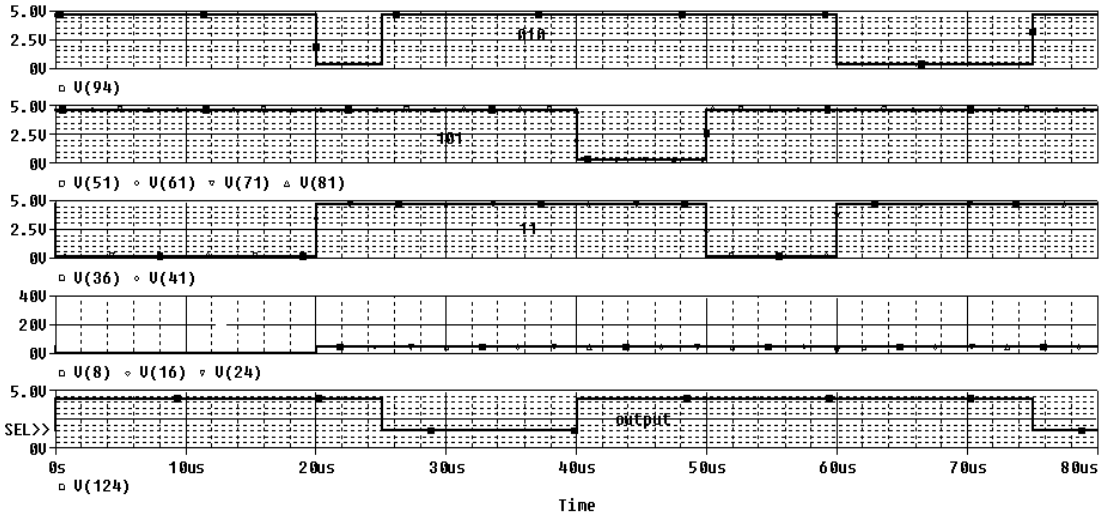


Fig 2.18 System Implementation of Insulin signal cascade using BiCMOS

2.4.1.4 TNF, EGF and Insulin signal cascade : The response of input signal (in the combination of TNF, EGF and Insulin) in presence of key players regulating different pathways by using CMOS is shown in Fig 2.19 and by using BiCMOS is shown in Fig 2.20. As the ten marker proteins are present (‘1’) then, it activates five different

pathways. V(31) is represented as output for CMOS and V(46) is represented as output for BiCMOS. Details of input notation for CMOS and BiCMOS are shown in Table 2.5.

Table 2.5 : Different key proteins involved in communicating signal through different pathways for CMOS and BiCMOS implementation

	Input		Combination of marker proteins
	CMOS	**BiCMOS**	
Pathway 1	V(5)	V(8)	RAF, MEK, ERK
Pathway 2	V(11)	V(16)	RAL, MEKK, JNK
Pathway 3	V(22)	V(42)	p38, MK2
Pathway 4	V(27)	V(38)	mTOR, PTEN, AkT
Pathway 5	V(17)	V(24)	RIP, IκB, NF-κB

Now to activate first pathway all the three marker proteins should be present (‘1’) leading to cell survival. Similarly for pathways 2, 4 and 5. But to activate pathway 3, marker protein PTEN should be absent (‘0’) than it will lead to cell survival.

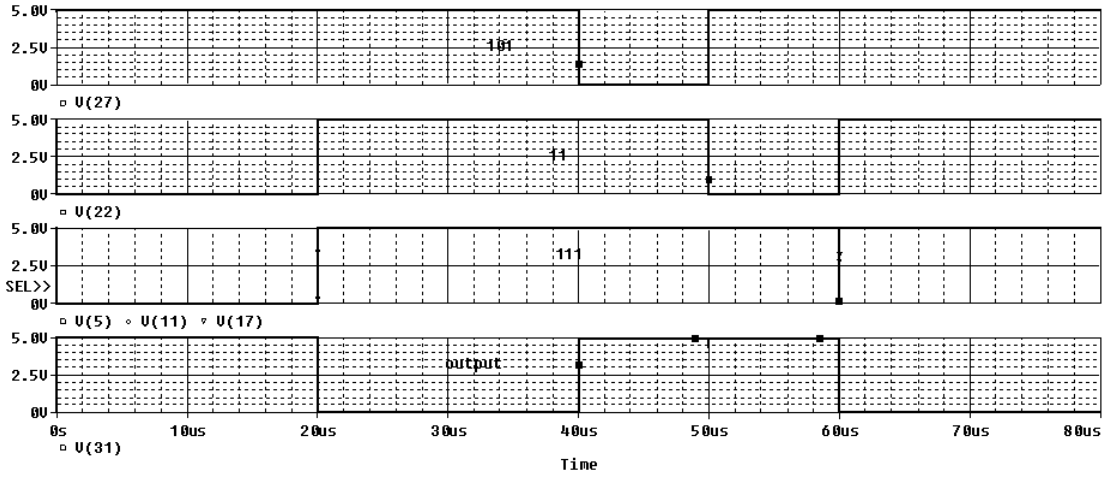


Fig 2.19 System Implementation of TNF, EGF and Insulin combined signal cascade using CMOS

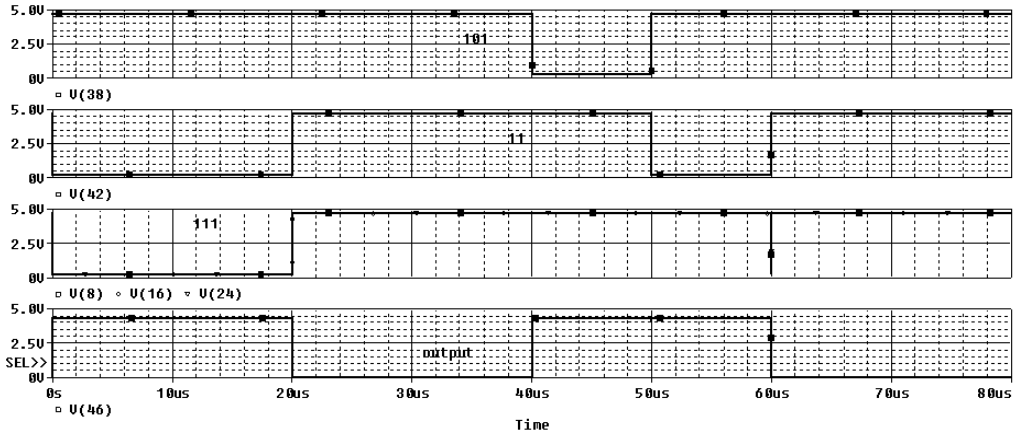


Fig 2.20 System Implementation of TNF, EGF and Insulin signal cascade using BiCMOS

2.5 SYSTEM IMPLEMENTATION USING HDL

VLSI stands for "Very Large Scale Integration". This is the field which involves packing more and more logic devices into smaller and smaller areas.

Without going into details, we can say that the VHDL, can be called as the "C" of the VLSI industry. VHDL stands for "VHSIC Hardware Definition Language", where VHSIC stands for "Very High Speed Integrated Circuit". This language is used to design the circuits at a high-level, in two ways. It can either be a behavioural description, which describes what the circuit is supposed to do, or a structural description, which describes what the circuit is made of. It is a hardware description language that can be used to model a digital system at many levels of abstraction, ranging from the algorithmic level to the gate level. The complexity of the digital system being modeled could vary from that of simple gate to the complete digital electronic system, or any thing in between. The digital system can also be described hierarchically. Timing can also be explicitly modeled in the same description. VHDL has many features appropriate for describing the behavior of electronic components ranging from simple logic gates to complete microprocessors and custom chips. Features of VHDL allow electrical aspects of circuit behavior (such as rise and fall times of signals, delays through gates, and functional operation) to be precisely described [70, 71]. The resulting VHDL simulation models can then be used as building blocks in larger circuits (using schematics, block diagrams or system-level VHDL descriptions) for the purpose of simulation.

VHDL is also a general-purpose programming language; just as high-level programming languages allow complex design concepts to be expressed as computer programs.

2.5.1 RESULTS AND DISCUSSION: We have implemented the system model regulating the binary decision of cell survival (‘1’) and cell death (‘0’) using VHDL and simulated with the help of Xilinx tool. Only the key players such as P13K, TNFR1, EGFR, IRS, IKK, Grb2, SOS, Ras, TRADD, Traf2 if present then the cell survive else the cell die.

2.5.1.1 TNF signal cascade : The response of input signal of TNF in presence of key players regulating different pathways. Table 2.6 shows the five different pathways, its input notation and output notation used to implement VHDL coding which is shown in Fig 2.21. As the ten marker proteins are present (‘1’) then, it activates five different pathways.

Table 2.6 Different key proteins involved in communicating signal through different pathways for VHDL implementation of TNF

	Input	**Combination of marker proteins**	**output**
Pathway 1	‘b’	RAF, MEK, ERK	‘k’
Pathway 2	‘c’	RAL, MEKK, JNK	‘l’
Pathway 3	‘e’	RIP, IκB, NFκB	‘n’
Pathway 4	‘f’	MAP3k, p38, MK2	‘o’
Pathway 5	‘g’	FAS	‘p’

Now to activate first pathway all the three marker proteins should be present (‘1’) than only it will lead to cell survival. Similarly for pathways 2, 3, and 4. But to activate pathway 5, marker protein FAS should be absent (‘0’) than it will lead to cell survival.

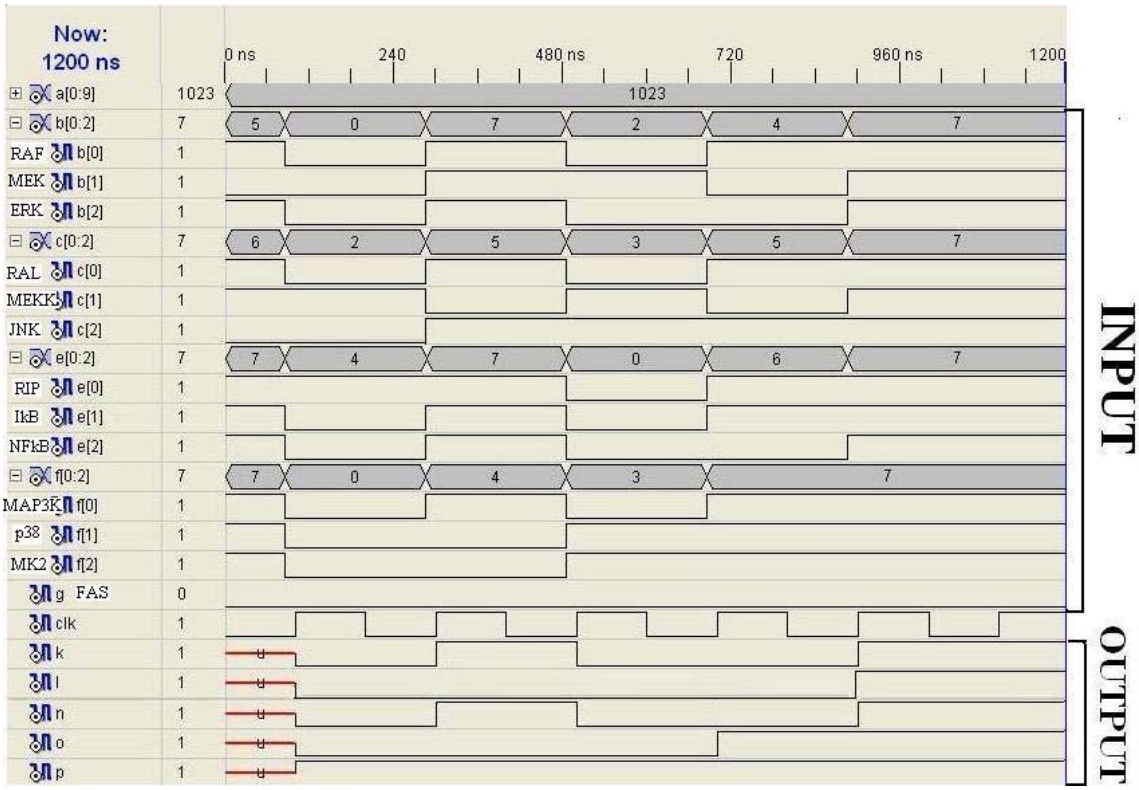


Fig 2.21 System Implementation of TNF signal cascade using VHDL

2.5.1.2 EGF signal cascade : The response of input signal of EGF in presence of key players regulating different pathways. Table 2.7 shows the nine different pathways, its input notation and output notation used to implement VHDL coding which is shown in Fig 2.22. As the ten marker proteins are present (‘1’) then, it activates nine different pathways.

Table 2.7 Different key proteins involved in communicating signal through different pathways for VHDL implementation of EGF

	Input	**Combination of marker proteins**	**output**
Pathway 1	‘b’	RAL, RAC, Rho	‘k’
Pathway 2	‘c’	RAL, MEKK, JNK	‘l’
Pathway 3	‘d’	RAF, MEK, ERK	‘m’
Pathway 4	‘f’	p38, MK2	‘o’
Pathway 5	‘g’	PTEN, AkT, NFκB	‘p’
Pathway 6	‘h’	PTEN, AkT, BAD	‘q’
Pathway 7	‘i’	PTEN, AkT, p53	‘r’
Pathway 8	‘j’	PTEN, AkT, FKHR	‘s’
Pathway 9	‘w’	JAK, STAT	‘y’

Now to activate first pathway, all the three marker proteins should be present (‘1’) than only it will lead to cell survival. Similarly for pathways 2, 3, 4 and 9 all the marker proteins should present. To activate pathway 5, 6 and 7 marker protein PTEN should be absent (‘0’) and to activate pathway 8 PTEN and FKHR should be absent (‘0’) than it will lead to cell survival.

2.5.1.3 Insulin signal cascade : The response of input signal of Insulin in presence of key players regulating different pathways. Table 2.8 shows the nine different pathways, its input notation and output notation used to implement VHDL coding which is shown in Fig 2.23. As the ten marker proteins are present (‘1’) then, it activates ten different pathways.

Now to activate first pathway, all the three marker proteins should be present (‘1’) than only it will lead to cell survival. Similarly for pathways 2, 3, 4, and 10 all the marker

proteins should present. To activate pathway 5, 6 7 and 9 marker protein PTEN should be absent (‘0’) and to activate pathway 8, PTEN and FKHR should be absent (‘0’) than it will lead to cell survival.

Table 2.8 Different key proteins involved in communicating signal through different pathways for VHDL implementation of Insulin

	Input	**Combination of marker proteins**	**output**
Pathway 1	‘b’	RAL, RAC, Rho	‘k’
Pathway 2	‘c’	RAL, MEKK, JNK	‘l’
Pathway 3	‘d’	RAF, MEK, ERK	‘m’
Pathway 4	‘f’	p38, MK2	‘o’
Pathway 5	‘g’	PTEN, AkT, NFκB	‘p’
Pathway 6	‘h’	PTEN, AkT, BAD	‘q’
Pathway 7	‘i’	PTEN, AkT, p53	‘r’
Pathway 8	‘j’	PTEN, AkT, FKHR	‘s’
Pathway 9	‘u’	PTEN, AkT, mTOR	‘t’
Pathway 10	‘w’	JAK, STAT	‘z’

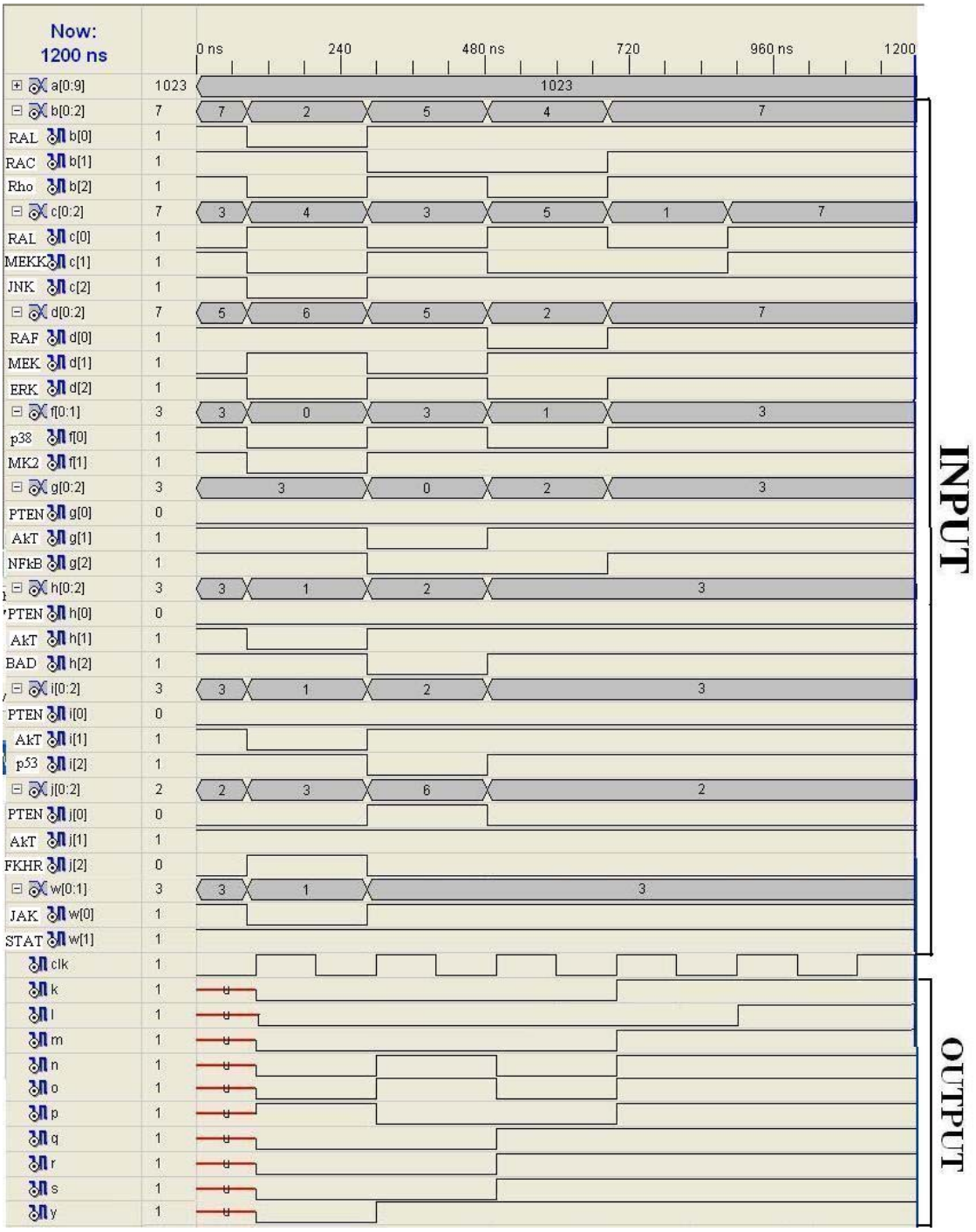


Fig 2.22 System Implementation of EGF signal cascade using VHDL

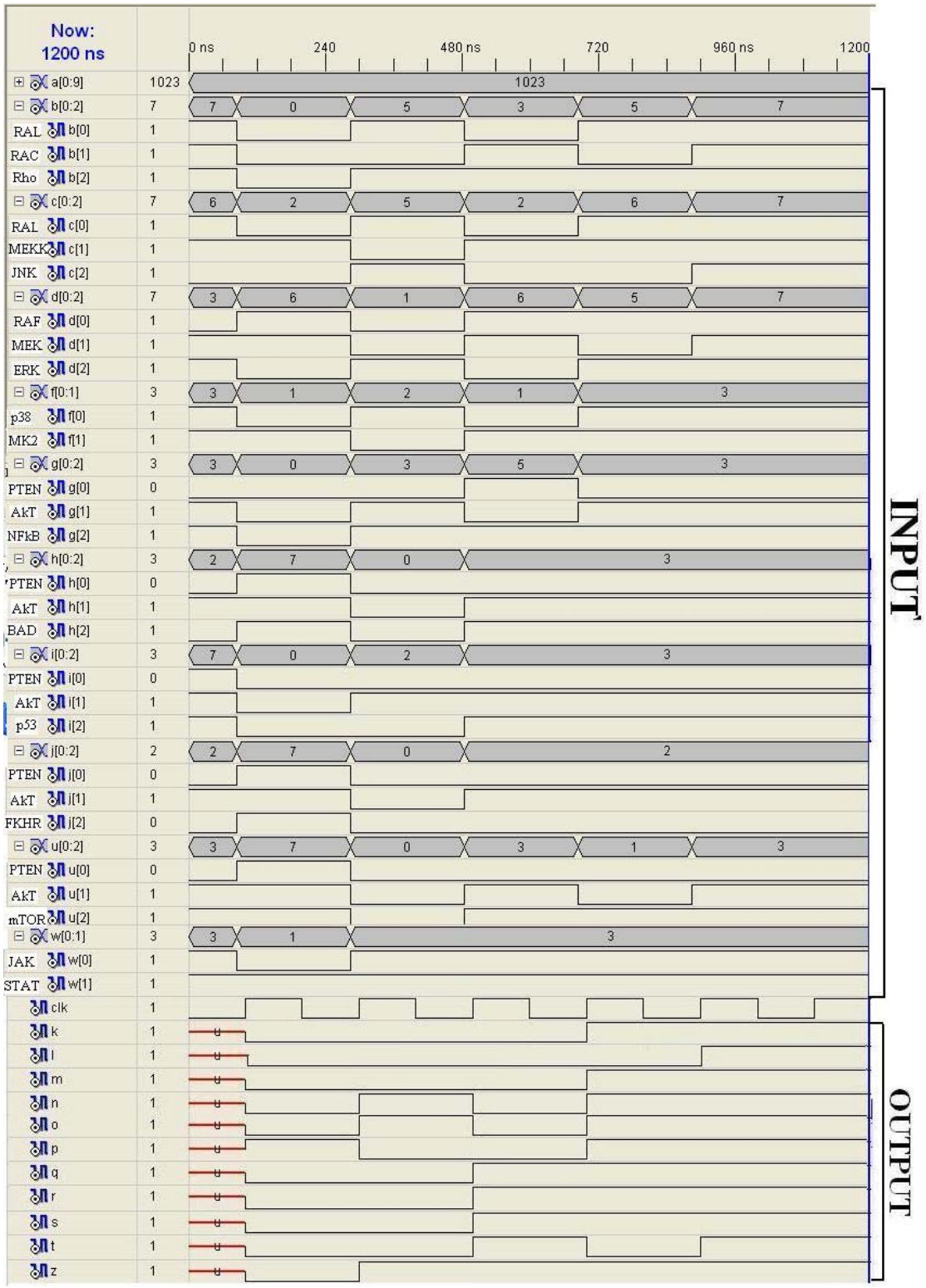


Fig 2.23 System Implementation of Insulin signal cascade using VHDL

2.5.1.4 TNF, EGF and Insulin combined signal cascade : The response of input signal (in the combination of TNF, EGF and Insulin) in presence of key players regulating different pathways. Table 2.9 shows the five different pathways, its input notation and output notation used to implement VHDL coding which is shown in Fig 2.24. As the ten marker proteins are present ('1') then, it activates five different pathways.

Table 2.9 Different key proteins involved in communicating signal through different pathways for VHDL implementation of TNF, EGF and Insulin combined

	Input	**Combination of marker proteins**	**output**
Pathway 1	'b'	RAF, MEK, ERK	'k'
Pathway 2	'c'	RAL, MEKK, JNK	'l'
Pathway 3	'd'	p38, MK2	'm'
Pathway 4	'e'	mTOR, PTEN, AkT	'n'
Pathway 5	'f'	RIP, IκB, NF-κB	'o'

Now to activate first pathway all the three marker proteins should be present ('1') than only it will lead to cell survival. Similarly for pathways 2, 4 and 5. But to activate pathway 3, marker protein PTEN should be absent ('0') than it will lead to cell survival.

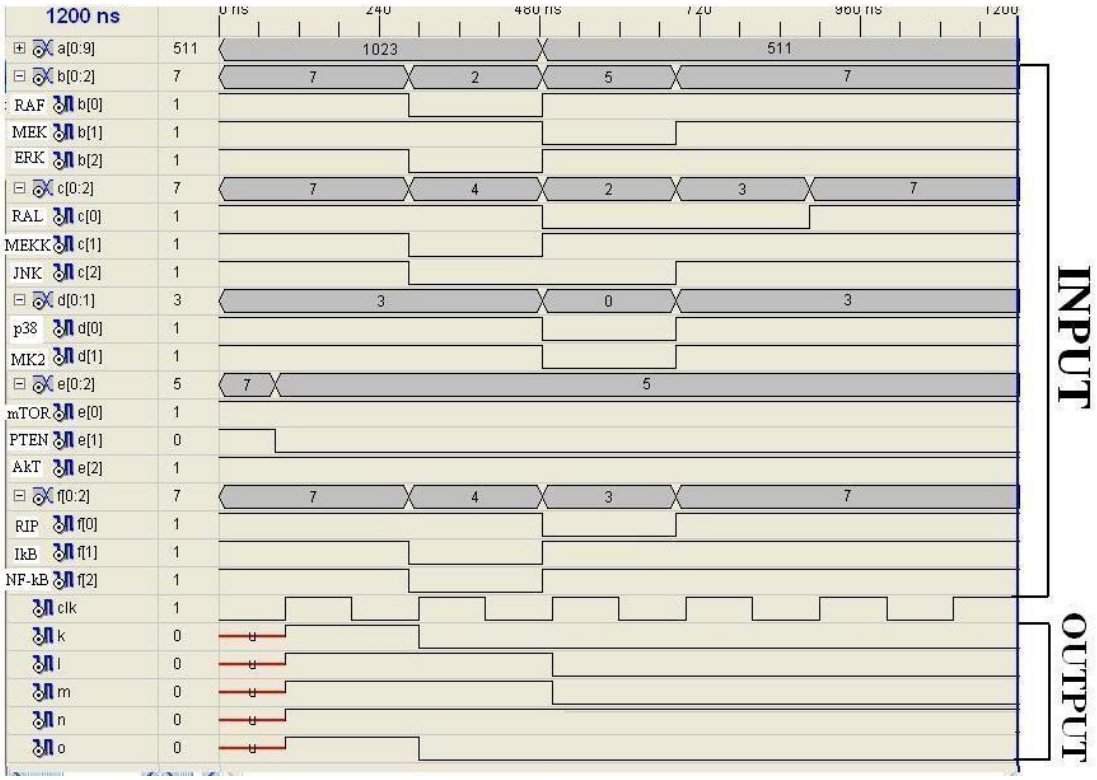


Fig 2.24 System Implementation of TNF, EGF and Insulin combined signal cascade using VHDL

2.6 SYSTEM IMPLEMENTATION USING FUZZY LOGIC

Fuzzy systems are an alternative to traditional notions of set membership and logic that has its origins in ancient Greek philosophy, and applications at the leading edge of Artificial Intelligence. Fuzzy logic has rapidly become one of the most successful of today's technologies for developing sophisticated control systems [72]. The reason for which is very simple. Fuzzy logic addresses such applications perfectly as it resembles human decision making with an ability to generate precise solutions from certain or approximate information.

Fuzzy Logic is a departure from classical two-valued sets and logic, that uses "soft" linguistic (e.g. large, hot, tall) system variables and a continuous range of truth values in the interval [0,1], rather than strict binary (True or False) decisions and assignments [73]. Formally, fuzzy logic is a structured, model-free estimator that approximates a function through linguistic input/output associations. Fuzzy rule-based systems apply these methods to solve many types of "real-world" problems, especially where a system is difficult to model, is controlled by a human operator or expert, or where ambiguity or vagueness is common. A typical fuzzy system consists of a rule base, membership functions, and an inference procedure.

FUZZY SET REPRESENTATION

A FUZZY SET is a set with a smooth boundary. Fuzzy set theory generalizes classical set theory to allow partial membership. The best way to introduce fuzzy sets is to start with a limitation of classical sets. A set in classical set theory always has the sharp boundary because membership in a set is a black and white concept- an object either completely belongs to the set or does not belong to the set at all.

Even though some sets do have sharp boundaries many others do not have sharp boundaries. Fuzzy set theory addresses this limitation by allowing membership in a set to be a matter of degree. The degree of membership in a set is expressed by a number between zero and one. Zero means entirely not in the set, one means completely in the set and number in between means partially in the set.

This way, a smooth and gradual transition from the regions outside the set to those in the set can be described. A fuzzy set is thus defined by a function that maps objects in a domain of concern to their membership value in the set. Such a function is called the membership function and is denoted by the Greek symbol (μ). Even though one may attempt to define a membership function of arbitrary shape it is strongly recommended to use parameterizable functions that can be defined by a small number of parameters. The parameterizable membership functions most commonly used in practice are the triangular membership functions and that trapezoid membership function. The former has three parameters and then later has four parameters. The common method of representing fuzzy set is

$$A = \{x, \mu_A(x)\} \quad x \in X$$

...(2.1)

where x is an element in X and $\mu_A(x)$ is the membership function of set, A which defines the membership of fuzzy set A in the universe of discourse, X. An alternative method to represent the singleton function is

$$A = \sum_{x_i \in X} \mu_A(x)/x_i$$

...(2.2)

The above representation is for the discrete universe of discourse. The fuzzy set representation for the continuous membership function is given by

$$A = \int_x \mu_A(x)/x_i$$

...(2.3)

BLOCK DIAGRAM OF FUZZY SYSTEM: In the Fuzzy Logic , there are five parts of the fuzzy inference process shown in Fig 2.25. Fuzzification of the input variables, application of the fuzzy operator (AND or OR) in the antecedent, implication from the antecedent to the consequent, aggregation of the consequents across the rules, and defuzzfication.

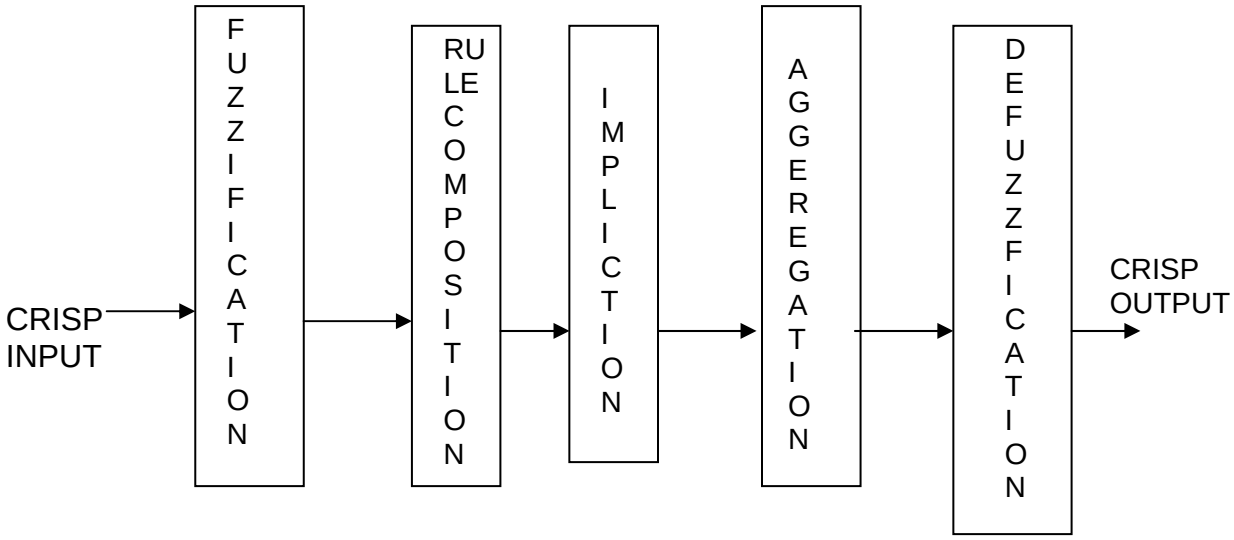


Fig 2.25 Fuzzy System Model

Step 1 : Fuzzification : The first step is to take the inputs and determine the degree to which they belong to each of the appropriate fuzzy sets via membership functions. In the Fuzzy Logic Toolbox, the input is always a crisp numerical value limited to the universe of discourse of the input variable and the output is a fuzzy degree of membership in the qualifying linguistic set (always the interval between 0 and 1).

Step 2 : Apply Fuzzy operator : Rule Composition : Once the inputs have been fuzzified, we know the degree to which each part of the antecedent has been satisfied for each rule. If the antecedent of a given rule has more than one part, the fuzzy operator is applied to obtain one number that represents the result of the antecedent for that rule. This number will then be applied to the output function. The input to the fuzzy operator is two or more membership values from fuzzified input variables. The output is a single truth value.

In the Fuzzy Logic Toolbox, two built in AND methods are supported : *min* (minimum) and *prod* (product). Two built in OR methods are also supported : *max* (maximum) and the probabilistic OR (*probor* or algebraic sum)

Step 3 : Implication : Before applying the implication method, we must take care of the rule's weight. Every rule has a weight (a number between 0 and 1), which is applied to

the number given by the antecedent. Generally this weight is 1 and so it has no effect at all on the implication process. From time to time we may want to weight one rule relative to the others by changing its weight value to something other than 1.

Once proper weighting has been assigned to each rule, the implication method is implemented. A consequent is a fuzzy set represented by a membership function, which weights appropriately the linguistic characteristics that are attributed to it. The consequent is reshaped using a function associated with the antecedent (a single number). The input for the implication process is a single number by the antecedent, and the output is a fuzzy set. Implication is implemented for each rule. Two built in methods are supported, and they are the same functions that are used by the AND method : *min* (minimum), which truncated the output fuzzy set, and *prod* (product), which scales the output fuzzy set.

Step 4 : Aggregation : Since decision are based on the testing of all of the rules in an FIS, the rules must be combined in some manner in order to make a decision. Aggregation is the process by which the fuzzy sets that represents the output variable, just prior to the fifth and final step, Defuzzification. The input of the aggregation process is the list of truncated output functions returned by the implication process for each rule. The output of the aggregation process is one fuzzy set for each output variable.

Notice that as long as the aggregation method is commutative, then the order in which the rules are executed is unimportant. Three built-in methods are supported : *max* (maximum), *probor* (probabilistic OR) and *sum* (simply the sum of each rule's output set).

Step 5 : Defuzzification : The input for the Defuzzification process is a fuzzy set (the aggregate output fuzzy set) and the output is a single number. As much as fuzziness helps the rule evaluation during the intermediate steps, the final desired output for each variable is generally a single number. However, the aggregate of a fuzzy set encompasses a range of output values and so must be defuzzified in order to resolve a single output value form the set. Perhaps the most popular Defuzzification method is the *centroid* calculation, which returns the center of area under the curve. There are five built in methods

supported : centroid, bisector, middle of maximum, largest of maximum and smallest of maximum.

Fuzzification Technique : Fuzzification is the process of changing a real scalar value into a fuzzy value. This is achieved with the different types of fuzzifiers.

Membership function of a Fuzzy set is defined as the characteristic function or curve of a fuzzy set, which assigns to each element in a Universe of Discourse a value between 0 and 1 defining its degree of presence in the fuzzy set and is known as the membership value and is designated as μ(x). We have mentioned different membership functions like sigmoid MF, Anti sigmoid MF, etc.

1. TRIANGULAR FUNCTION

The equation for triangular membership function is shown in Eq.2.4. Points *a*, *b*, *c*, and *x* are defined in Fig 2.26.

$$\mu(x)=\begin{cases} 0, & \text{when } x \le a \\ \dfrac{(x-a)}{(b-a)}, & \text{when } a \le x \le b \\ \dfrac{(c-x)}{(c-b)}, & \text{when } b \le x \le c \\ 0, & \text{when } x > c \end{cases} \qquad \text{...(2.4)}$$

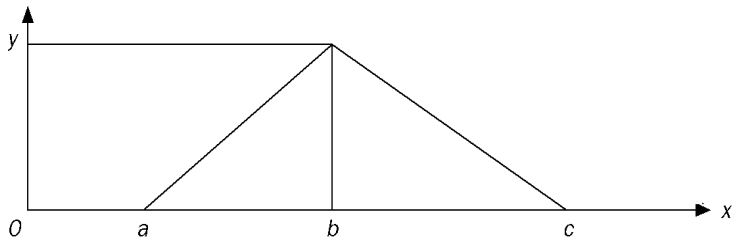


Fig 2.26 Showing parameter *a*, *b*, and *c*, and variable *x* point of equation of Triangular function

2. TRAPEZOIDAL FUNCTION

The trapezoidal membership function has a flat top with membership value of 1 for a small range about the central point of the function. It eliminates the problems associated with the triangular membership function. The equation for

trapezoidal membership function is shown in Eq 2.5. The variable x and the parameters a, b, c and d are shown in Fig 2.27.

$$\mu_A(x) = \begin{cases} 0, & \text{when } x < a \text{ or } x > d \\ \dfrac{(x-a)}{(b-a)}, & \text{when } a \le x \le b \\ 1, & \text{when } b \le x \le c \\ \dfrac{(d-x)}{(d-b)}, & \text{when } c \le x \le d \end{cases}$$

...(2.5)

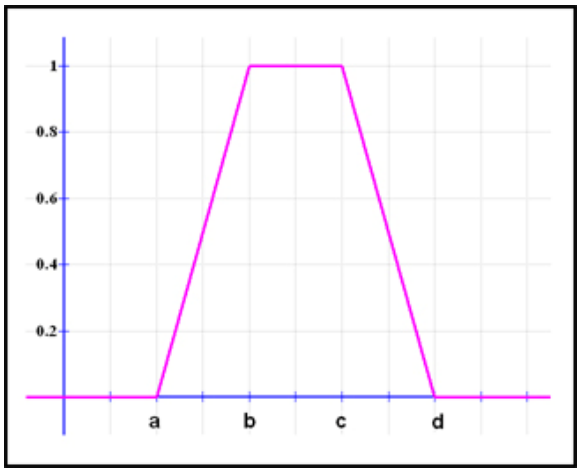


Fig 2.27 Showing parameter a, b, c, and d point of equation of Trapezoidal function

3. SIGMOIDAL FUNCTION

The Sigmoidal membership function is defined by parameter: '*a*' defines the slope of the curve (Eq 2.6) and also the sign of the parameter 'a' decides whether the curve will be right-open or left-open shown in Fig 2.28. Also parameter '*b*' defines the point $\mu_A(b) = 0.5$. It can be defined as,

$$\mu_A(x) = \frac{1}{1 + e^{-a(x-c)}}$$

...(2.6)

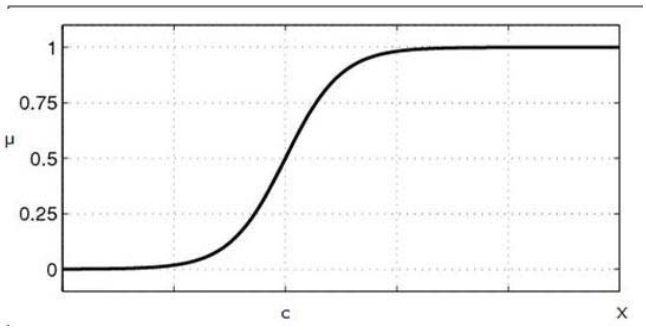


Fig 2.28 Showing Sigmoidal Function

Knowledge Base : The knowledge base is the module around which the expert system is built. It contains the formal representation of the information provided by the domain expert. This information may be in the form of problem-solving rules, procedures, or data intrinsic to the domain.It contains application domain and procedural knowledge. It consists of data base and rule base.

Data Base

1. It provides the necessary definitions which are used to define linguistic variable of control roots.
2. It gives the choice of membership function.
3. It provides the fuzzy partitioning between input and output space.

Rule Base :

1. It is the condition in which antecedent is process variable and consequent is control output variable.
2. It gives the choice of process state variables.
3. It gives the different type of control rules.

Fuzzy Implication : The process of shaping of the consequent variable based upon antecedent is called implication. As discussed earlier, the If-Then rules for fuzzy logic can be written as If <fuzzy proposition> Then <fuzzy proposition>. The propositional variables A and B are replaced by fuzzy propositions, and the implication can be replaced by fuzzy *union*, fuzzy *intersection* and fuzzy *complement*. There are many fuzzy implications**:**

1. Mamdani Style

2. Larson Style

Mamdani Min Implication : Mamdani proposed a fuzzy implication rule for fuzzy control in 1977. It is a simplified version of Zadeh implication operator expressed in Eq. 2.7.

$$\phi\left[\mu_A(x), \mu_B(x)\right] \equiv \mu_A(x) \wedge \mu_B(x) \qquad ...(2.7)$$

Larsen Product Implication : The Larsen product implication is given by Eq. 2.8.

$$\phi\left[\mu_A(x), \mu_B(x)\right] \equiv \mu_A(x).\mu_B(x) \qquad ...(2.8)$$

Defuzzification Technique : Fuzzy logic is a rule-based system in which the input to the fuzzy system is a scalar value that is fuzzified. The set of rules is applied to the fuzzified input. The output of each rule is fuzzy. These fuzzy outputs need to be converted into a scalar output quantity so that the nature of the action to be performed can be determined by the system. The process of converting the fuzzy output is called Defuzzification. Before an output is defuzzified all the fuzzy outputs of the system are aggregated with a union operator which can be expressed as Eq. 2.9

$$\mu_A = \bigcup_i (\mu_i(x)) \qquad ...(2.9)$$

There are many Defuzzification techniques but primarily only three of them are in common use. These Defuzzification techniques are

1**. Maximum Defuzzification Technique :** This method gives the output with the highest membership function. This Defuzzification technique is very fast but is only accurate for peaked output. This technique is given by algebraic expression as

$$\mu_A(x^*) \geq \mu_A(x) \qquad ...(2.10)$$

where x^* is the defuzzified value. This is shown graphically in Fig. 2.29.

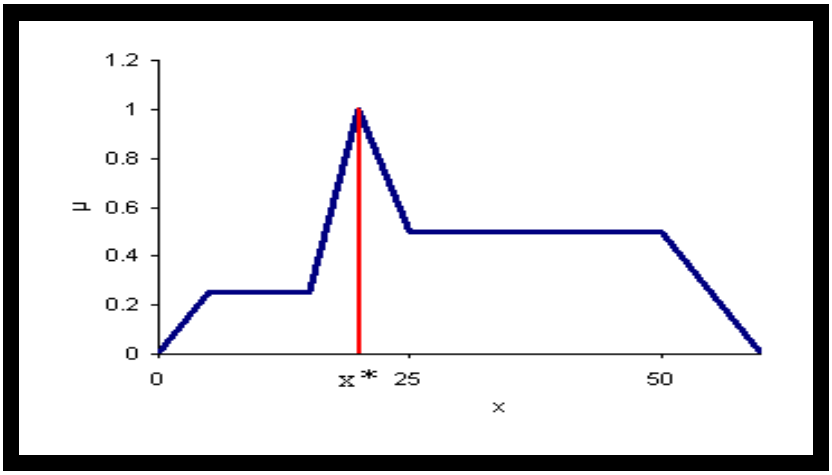


Fig 2.29 Max-membership Defuzzification methods

2. **Centroid Defuzzification Technique :** This method is also known as center of gravity or center of area Defuzzification. This technique was developed by Sugeno in 1985. This is the most commonly used technique and is very accurate. The Centroid Defuzzification technique can be expressed as

$$x^* = \frac{\int \mu_i(x)\, x\, dx}{\int \mu_i(x)\, dx}$$

...(2.11)

where x^* is the defuzzified output, $\mu_I(x)$ is the aggregated membership function and x is the output variable. The only disadvantage of this method is that it is computationally difficult for complex membership functions.

3. **Weighted Average Defuzzification Technique :** In this method the output is obtained by the weighted average of each output of the set of rules stored in the knowledge base of the system. The weighted average Defuzzification technique can be expressed as

$$x^* = \frac{\sum_{i=1}^{n} m^i\, w_i}{\sum_{i=1}^{n} m^i}$$

...(2.12)

where x^* is the defuzzified output, m^i is the membership of the output of each rule, and w_i is the weight associated with each rule. This method is computationally faster and easier and gives fairly accurate result.

2.6.1 RESULTS AND DISCUSSION :

Among logic-based methods, the simplicity of Boolean models makes them attractive as a means to render biological networks. For example, a discrete-state representation of the level of phosphorylation of JNK might use three input edges TNF, TRAF2 and MAPK (where '1' means present or active, and '0' absent or inactive; Figure 2.30(*a*)). In Boolean logic, interactions among inputs are cast as combinations of elementary 'AND' gate that generate logic rules such as '(TNF AND TRAF2 AND MAPK)' and are most easily specified using gates (Figure 2.30(*b*)) and truth tables. Truth tables consist of lookup values for the outputs (consequent value) based on all possible combinations of input values (antecedents). Despite the appeal of Boolean models a two-state ''on-off'' representation of many biological signals is quite unrealistic. Working with FL models involves manipulating logic gates based on several adjustable parameters: (*i*) Membership functions (MFs) are used to assign values of inputs to a descriptive input class. (*ii*) MFs define the degree of membership (DOM) that quantifies the mapping between inputs and MFs and is always between 0 (no membership) and 1 (full membership). Fuzzy logic is so-named because inputs can have non-zero DOM to more than one MF, unlike discrete-state logic in which MFs and DOMs only take on values of 0 and 1. Figure 2.30(*c*) illustrates example MFs for Boolean and fuzzy logic models. (*iii*) The steepness of the membership functions is parameterized by the degree of fuzziness (note that Boolean logic models have a degree of fuzziness of 0). (*iv*) Logic rules relate the input state to the output state. In doing so, these rules encode how the input proteins regulate the activity of output protein. Once the logic rules are established, an FL gate is generated by first fuzzifying the inputs, a step that computes the DOM of each input state over the current input values and the pre-specified MFs. The degree of firing (DOF), then specifies whether a rule should be used (1) or not (0) as determined from the lowest DOM amongst the antecedents and the rule weight, a value between 0 and 1 that allows additional tuning of a rule's importance. In contrast to Boolean logic (BL) gates in which

only one rule can fire for any set of input values (that is, only one row in the truth table is applied) FL gates allow multiple rules to fire to varying degrees (as defined by the DOF, Figure 2.30(*d*)). Defuzzification is the final step in which the superposition of multiple rules is resolved to determine the output value for the gate.

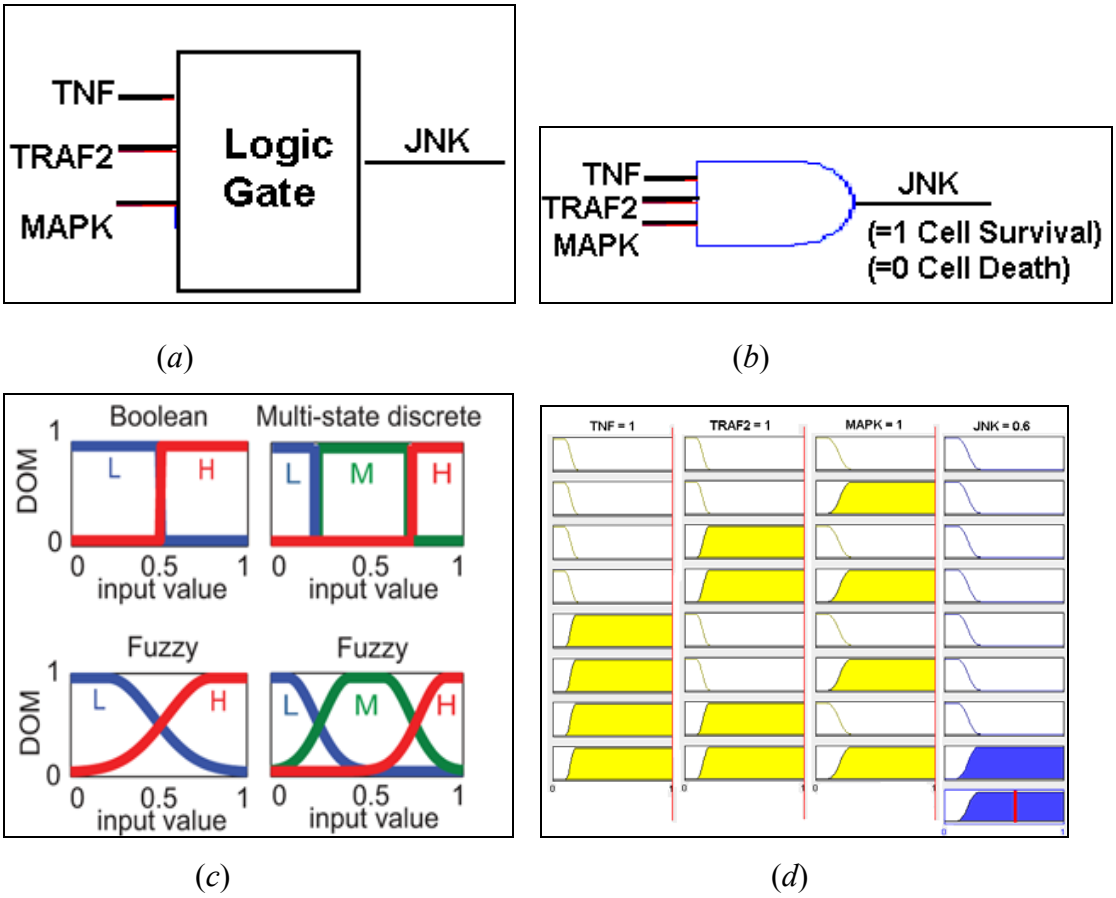


Fig 2.30 Fuzzy Logic Implementation (*a*) Logic-based models use incoming edges to contain activity level of input or regulatory network species (for JNK, the inputs were TNF, TRAF2, and MAPk) with the logic gate at the node that performs the logic operation to update output signal (JNK) (*b*) A Boolean logic gate for JNK could be represented in terms of the logic statement ‘‘(TNF and TARF2 and MAPK)’’, represented here in schematic form where the shape is an ‘‘AND-gate’’). (*c*) To set up a FL gate, the first step is to assign membership functions (MFs) to the input variables. Each input variable has two or three membership functions (‘L’, ‘M’, and ‘H’ representing low, medium, and high states, respectively). An MF relates an input value to that state’s degree of membership (DOM). MFs for Fuzzy and Boolean (2 MFs)/discrete multi-state (.2 MFs) logic forms are illustrated with the same state thresholds. (*d*) To set up a FL gate, the MFs for the inputs and for the outputs are defined. For simplicity, we use normalized input and output values. Next, logic rules are listed as ‘‘if A (the antecedent), then B (the consequent)’’ using the input and output states as descriptors. Weights between 0 and 1 are assigned to each rule, which is helpful for rules that should have minor influence. The rules for TNF, TRAF2, MAPK are each graphically listed with the outline of the membership functions specified for that rule’s antecedent. The consequent for each rule is also indicated by MF.

2.6.1.1 TNF cascade : The pathways involving TNF that regulates cell survival and cell cell death are as follows

1) $TNF / TRAF2 / MAPK \rightarrow JNK$ ($=1$ Cell Survival, $=0$ Apoptosis); (Result Shown in Fig 3.31(a))

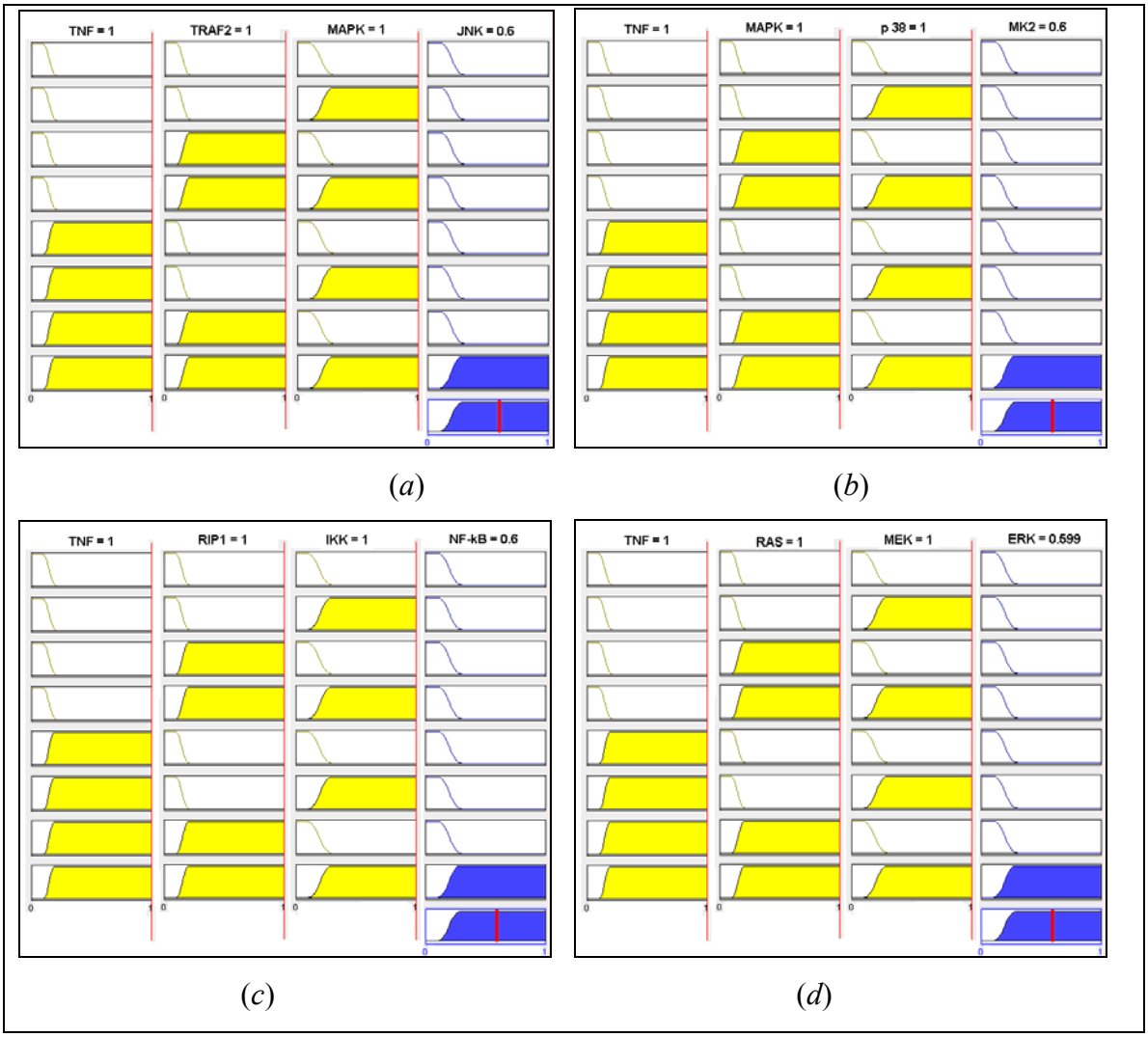
2) $TNF / MAPK / p38 \rightarrow MK2$ ($=1$ Cell Survival, $=0$ Apoptosis); (Result Shown in Fig 3.31(b))

3) $TNF / RIP1 / IKK \rightarrow NF\kappa B$ ($=1$ Cell Survival, $=0$ Apoptosis); (Result Shown in Fig 3.31(c))

4) $TNF / RAS / MEK \rightarrow ERK$ ($=1$ Cell Survival, $=0$ Apoptosis); (Result Shown in Fig 3.31(d))

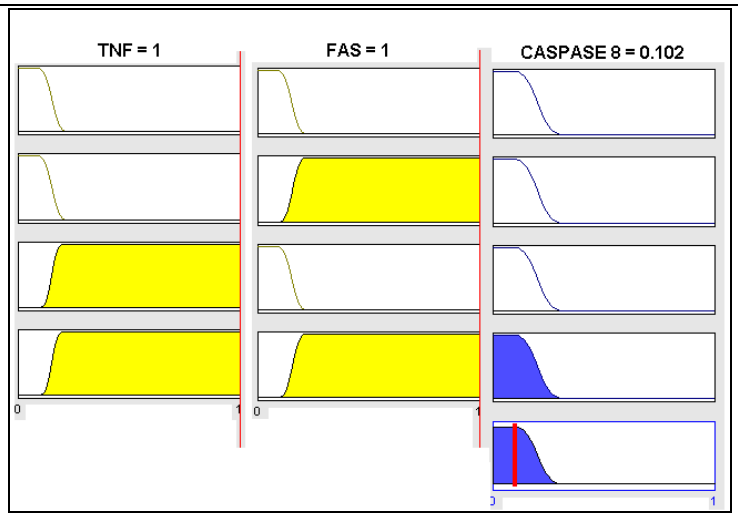
5) $TNF / FAS \rightarrow CASPASE\,8$ (Apoptosis). (Result Shown in Fig 3.31(e))

To get the output of all the above pathways of TNF, we have used Fuzzy Tool box of MATLAB by taking data as: Type ='Mamdani', And Method ='Min', Or Method ='Max', Implication method ='Min', Aggregation Method ='Max', Defuzzification Method ='Centroid' shown in Fig 3.31 (*a*-*e*). Yellow filled boxes are treated as '1' i.e. high, while blank ones are '0' i.e. low. In last column, blue filled part in Fig. 3.31 (*a*, *b*, *c*, *d*) represents Cell Survival while Fig 3.31(*e*) represents Cell death.



(*a*) (*b*)

(*c*) (*d*)



(*e*)

Fig 2.31 System Implementation of TNF signal cascade using Fuzzy (*a*) Output for TNF/ TRAF2/ MAPK = JNK pathway. (*b*) Output for TNF/ MAPK/ p38 = MK2 pathway. (*c*) Output for TNF/ RIP1/ IKK = NF-κB pathway. (*d*) Output for TNF/ RAS/ MEK = ERK pathway. (*e*) Output for TNF/ FAS = Caspase 8 pathway.

2.6.1.2EGF cascade : Following are the pathways of EGF regulating cell survival/ cell cell death . To get the output of all the above pathways of EGF, we have used Fuzzy Tool box of MATLAB by taking data as: Type ='Mamdani', And Method ='Min', Or Method ='Max', Implication method ='Min', Aggregation Method ='Max', Defuzzification Method ='Centroid' shown in Fig 3.32 (*a*-*g*). Yellow filled boxes are treated as '1' i.e. high, while blank ones are '0' i.e. low. In last column, blue filled part in Fig 3.32(*a*, *b*, *c*, *d*, *f*, *g*) represents Cell Survival while Fig 3.32 (*e*) represents cell death.

1) $EGF / RAS / MEK \rightarrow ERK (=1 \text{Cell Survival}, =0 \text{ Apoptosis});$ $(\text{Result Shown in Fig } 3.32(a))$

2) $EGF / RAS / MEKK1 \rightarrow JNK (=1 \text{Cell Survival}, =0 \text{ Apoptosis});$ $(\text{Result Shown in Fig } 3.32(b))$

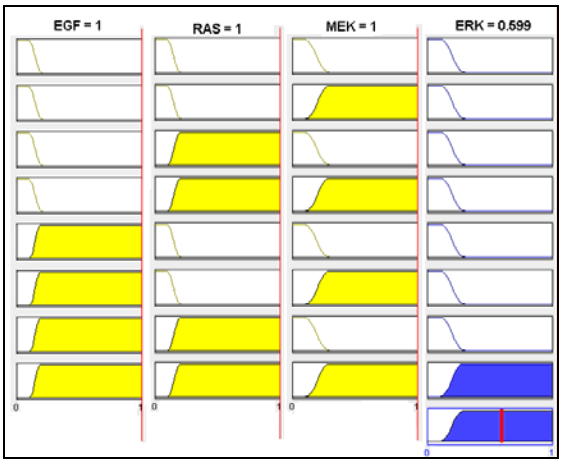
3) $EGF / PI3K / AKT \rightarrow NF\kappa B (=1 \text{Cell Survival}, =0 \text{ Apoptosis});$ $(\text{Result Shown in Fig } 3.32(c))$

4) $EGF / PI3K / AKT \rightarrow BAD (=1 \text{Cell Survival}, =0 \text{ Apoptosis});$ $(\text{Result Shown in Fig } 3.32(d))$

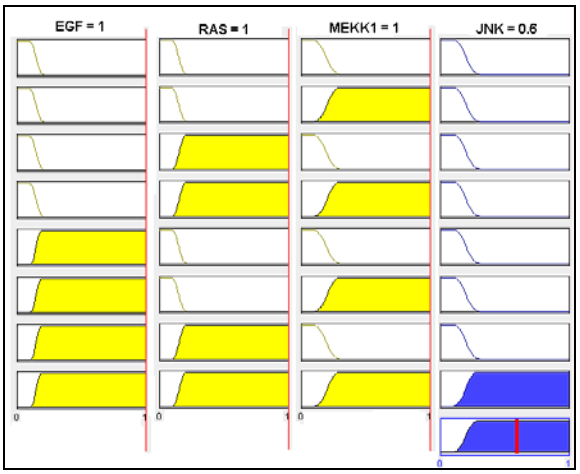
5) $EGF / PI3K / AKT \rightarrow FKHR (\text{ Apoptosis});$ $(\text{Result Shown in Fig } 3.32(e))$

6) $EGFR / JAK \rightarrow STAT (=1 \text{Cell Survival}, =0 \text{ Apoptosis});$ $(\text{Result Shown in Fig } 3.32(f))$

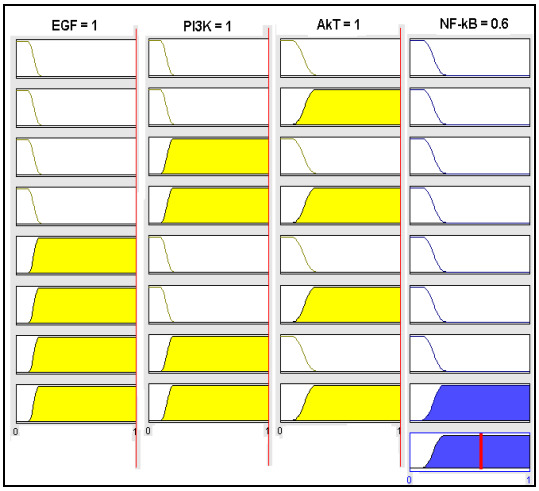
7) $\text{EGFR}/ p38 \rightarrow MK2 (=1 \text{Cell Survival}, =0 \text{ Apoptosis}).$ $(\text{Result Shown in Fig } 3.32(g))$


EGF = 1
RAS = 1
MEK = 1
ERK = 0.599

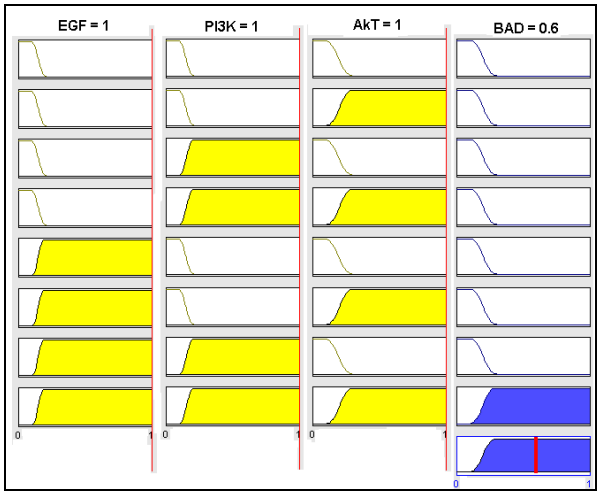
(*a*)


EGF = 1
RAS = 1
MEKK1 = 1
JNK = 0.6

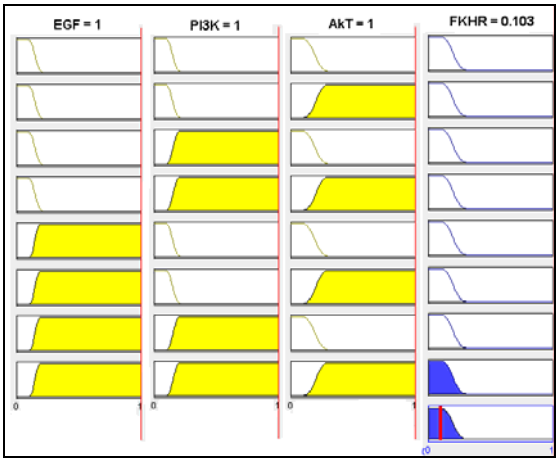
(*b*)


EGF = 1
PI3K = 1
AkT = 1
NF-kB = 0.6

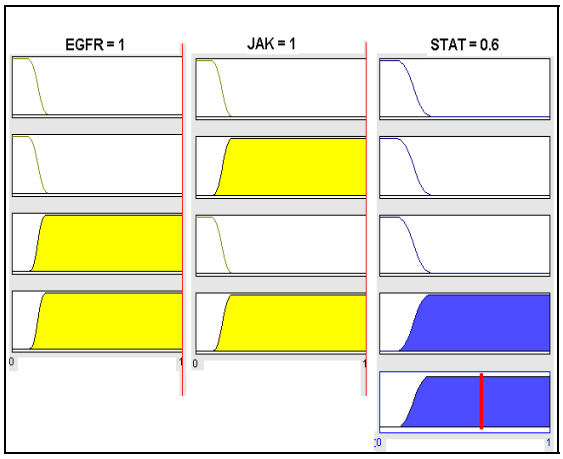
(*c*)


EGF = 1
PI3K = 1
AkT = 1
BAD = 0.6

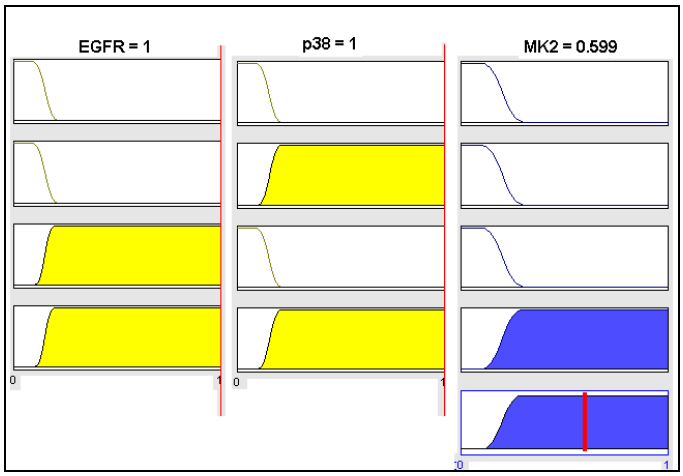
(*d*)


EGF = 1
PI3K = 1
AkT = 1
FKHR = 0.103

(*e*)


EGFR = 1
JAK = 1
STAT = 0.6

(*f*)



(*g*)

Fig 2.32 System Implementation of EGF signal cascade using Fuzzy (*a*) Output for EGF/ RAS/ MEK = ERK pathway; (*b*) Output for EGF/ RAS/ MEKK1= JNK pathway; (*c*) Output for EGF/PI3K/ AkT = NF-κB pathway; (*d*) Output for EGF/PI3K/ AkT = BAD pathway; (*e*) Output for EGF/PI3K/ AkT = FKHR pathway; (*f*) EGFR/ JAK = STAT Pathway; (g) EGFR/ p38 = MK2 Pathway.

2.6.1.3 Insulin Cascade : To get the output of all the above pathways of Insulin, we have used Fuzzy Tool box of MATLAB by taking data as: Type ='Mamdani', And Method ='Min', Or Method ='Max', Implication method ='Min', Aggregation Method ='Max', Defuzzification Method ='Centroid' shown in Fig 3.33 (*a-h*). Yellow filled boxes are treated as '1' i.e. high, while blank ones are '0' i.e. low. In last column, blue filled part in Fig 3.33(*a*, *b*, *c*, *d*, *f*, *g*, *h*) represents Cell Survival while Fig 3.33 (*e*) represents Cell death.

Pathways which lead to cell survival/death using INSULIN are as follows:

1) $Insulin / RAS / MEK \rightarrow ERK (=1 \text{Cell Survival}, =0 \text{ Apoptosis});$ $(\text{Result Shown in Fig } 3.33(a))$

2) $Insulin / RAS / MEKK1 \rightarrow JNK (=1 \text{Cell Survival}, =0 \text{ Apoptosis});$ $(\text{Result Shown in Fig } 3.33(b))$

3) $Insulin / PI3K / AKT \rightarrow NF\kappa B (=1 \text{Cell Survival}, =0 \text{ Apoptosis});$ $(\text{Result Shown in Fig } 3.33(c))$

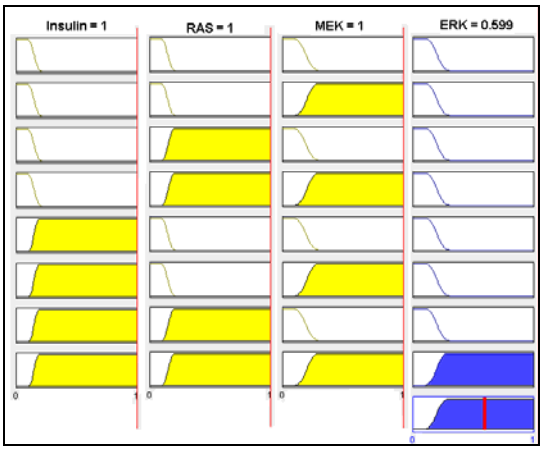
4) $Insulin / PI3K / AKT \rightarrow BAD (=1 \text{Cell Survival}, =0 \text{ Apoptosis});$ $(\text{Result Shown in Fig } 3.33(d))$

5) $Insulin / PI3K / AKT \rightarrow FKHR (\text{ Apoptosis});$ $(\text{Result Shown in Fig } 3.33(e))$

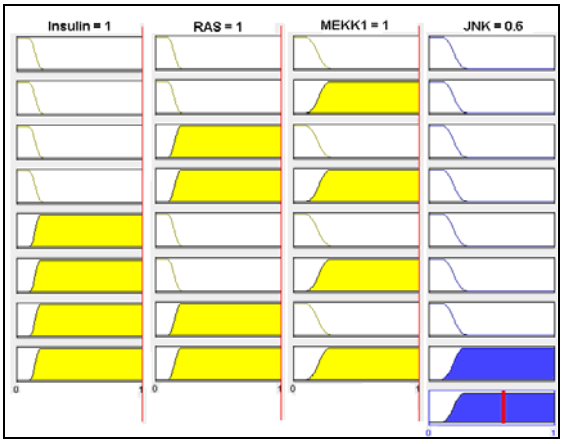
6) $IRS / JAK \rightarrow STAT (=1 \text{Cell Survival}, =0 \text{ Apoptosis});$ $(\text{Result Shown in Fig } 3.33(f))$

7) $IRS/\ p38 \rightarrow MK2 (=1 \text{Cell Survival}, =0 \text{ Apoptosis});$ $(\text{Result Shown in Fig } 3.33(g))$

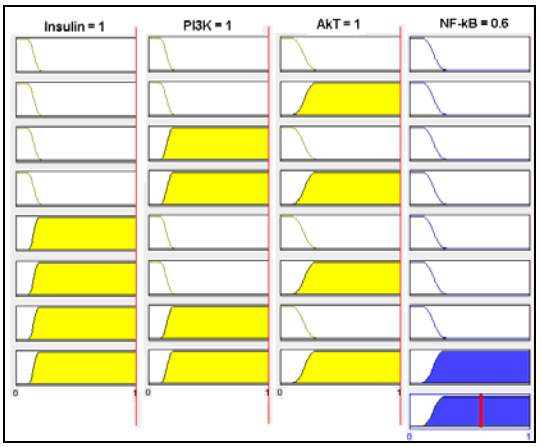
8) $Insulin / AKT / mTOR \rightarrow IRS (=1 \text{Cell Survival}, =0 \text{ Apoptosis}).$ $(\text{Result Shown in Fig } 3.33(h))$


Insulin = 1
RAS = 1
MEK = 1
ERK = 0.599

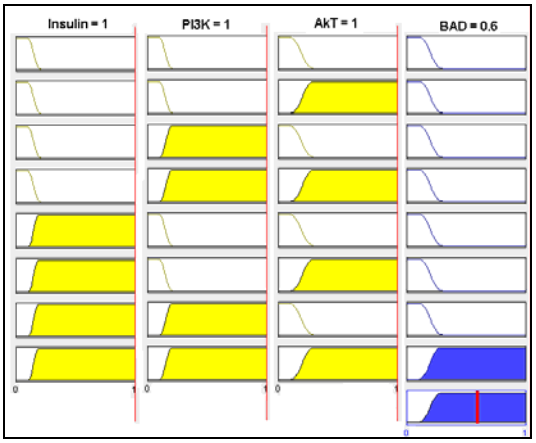
(*a*)


Insulin = 1
RAS = 1
MEKK1 = 1
JNK = 0.6

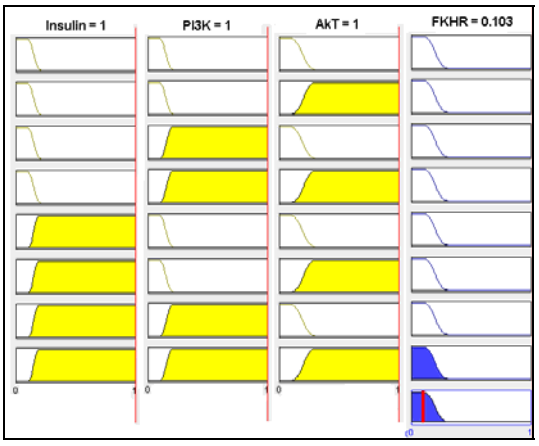
(*b*)


Insulin = 1
PI3K = 1
AkT = 1
NF-kB = 0.6

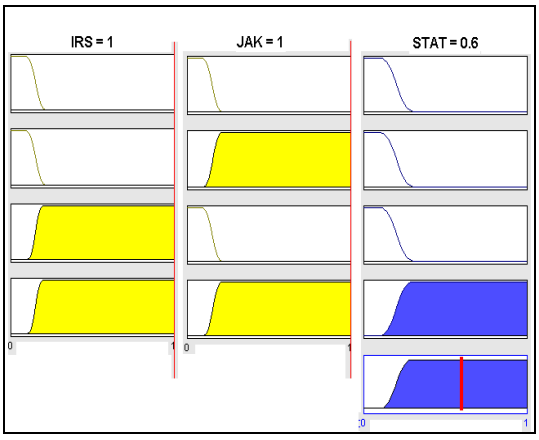
(*c*)


Insulin = 1
PI3K = 1
AkT = 1
BAD = 0.6

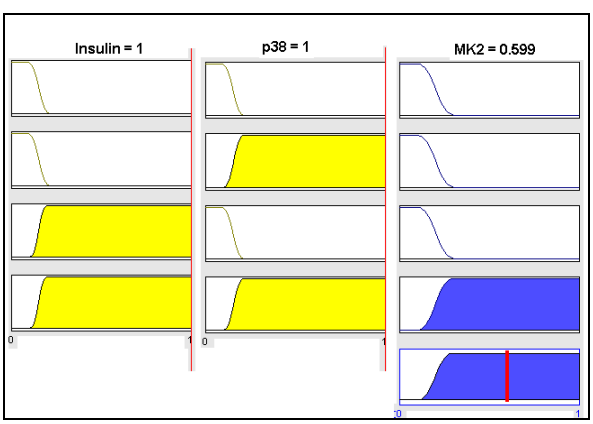
(*d*)


Insulin = 1
PI3K = 1
AkT = 1
FKHR = 0.103

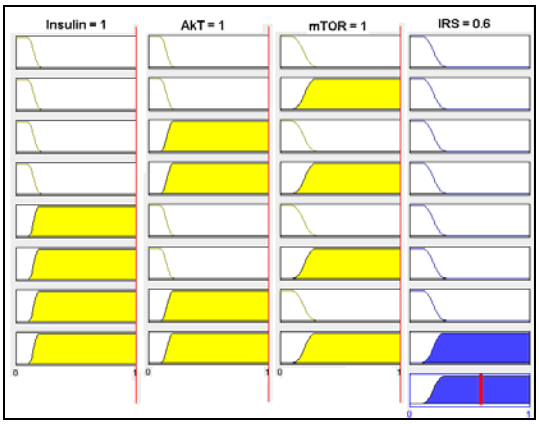
(*e*)


IRS = 1
JAK = 1
STAT = 0.6

(*f*)



(*g*)



(*h*)

Fig 2.33 System Implementation of Insulin signal cascade using Fuzzy. (*a*) Output for Insulin/ RAS/ MEK = ERK pathway; (*b*) Output for Insulin / RAS/ MEKK1= JNK pathway; (*c*) Output for Insulin/PI3K/ AkT = NF-κB pathway; (*d*) Output for Insulin/PI3K/ AkT = BAD pathway; (*e*) Output for Insulin/PI3K/ AkT = FKHR pathway; (*f*) IRS/ JAK = STAT Pathway; (*g*) Insulin/ p38 = MK2 Pathway; (*h*) Insulin/ AkT /mTOR = IRS Pathway.

CHAPTER 3
MATHEMATICAL MODELING OF CELL SURVIVAL AND CELL DEATH

The experimental observation of cell survival/ cell death treated with (TNF), in combination with epidermal growth factor (EGF) or insulin, were obtained from Gaudet *et al* (2005). The fate of cell decision is depends on the concentration of eleven marker proteins such as Mitogen-activated protein kinase-activated protein kinase 2 (MK2), c-jun N-terminal kinases(JNK), Forkhead transcription factor (FKHR), Mitogen-activated protein kinase and extracellular-regulated kinase (MEK), Extracellular-regulated kinase (ERK), Insulin receptor substrate (IRS), AkT, IKK, Phospho-to-total EGFR (ptEGFR), Phospho-to-total Akt (ptAkt), pAkT. Therefore, it is possible to built self consistent compendia cellular response based on the above eleven proteins that can be simulated computationally to yield important insights into the control of cell survival/ cell death. In this study we have implemented two computational models for the prediction of cell survival/ cell death utilizing the concentration of eleven proteins as follows :

1. System Implementation using Non Linear Modeling (Artificial Neural Network)

2. System Implementation using Linear Modeling (Deterministic Modeling)

3.1 SYSTEM IMPLEMENTATION USING NON-LINEAR MODELING (ARTIFICIAL NEURAL NETWORK)

An artificial neural network (ANN) model was developed for the prediction of cell survival/ cell death considering eleven marker proteins. For training the ANN model [74, 75, 76] experimental data form ten different concentrations of each marker proteins was taken as input, and their corresponding possible experimental output. We have implemented the Neural Network model using STATISTICA data miner software. It consists of two layers of multiple layer perceptrons (MLP) [77, 78]. The first layer consists of 11 input nodes, 6 hidden nodes and 4 output nodes, whereas the second layer

of Neural Network consists of 4 input nodes, 9 hidden nodes and 1 output node. If the predicted output in the second Neural Network is > 0.5 it will lead to cell survival otherwise it lead to cell death. The data from the four treatments were used as test set to validate the predictive accuracy of ANN model.

3.1.1 RESULTS AND DISCUSSION : We have developed two layer ANN for the prediction of cell survival/ cell death based on the concentration of eleven marker proteins as input. The first layer consists of one input layer with eleven node, where each node corresponding to the marker protein. It consists of one hidden layer with 6 hidden nodes and one output layer with 4 nodes. The first layer of ANN predicts different physiological state of the cell; (1) PS exposure, (2) Membrane permeability, (3) caspases cleavage and (4) DNA fragmentation shown in Fig 3.1. The output of first layer of ANN is the input to second layer of ANN that ultimately predicts the state of cell survival / cell death. The results reveal that ANN model is most adequate to estimate the physiological functions from intracellular protein expressions.

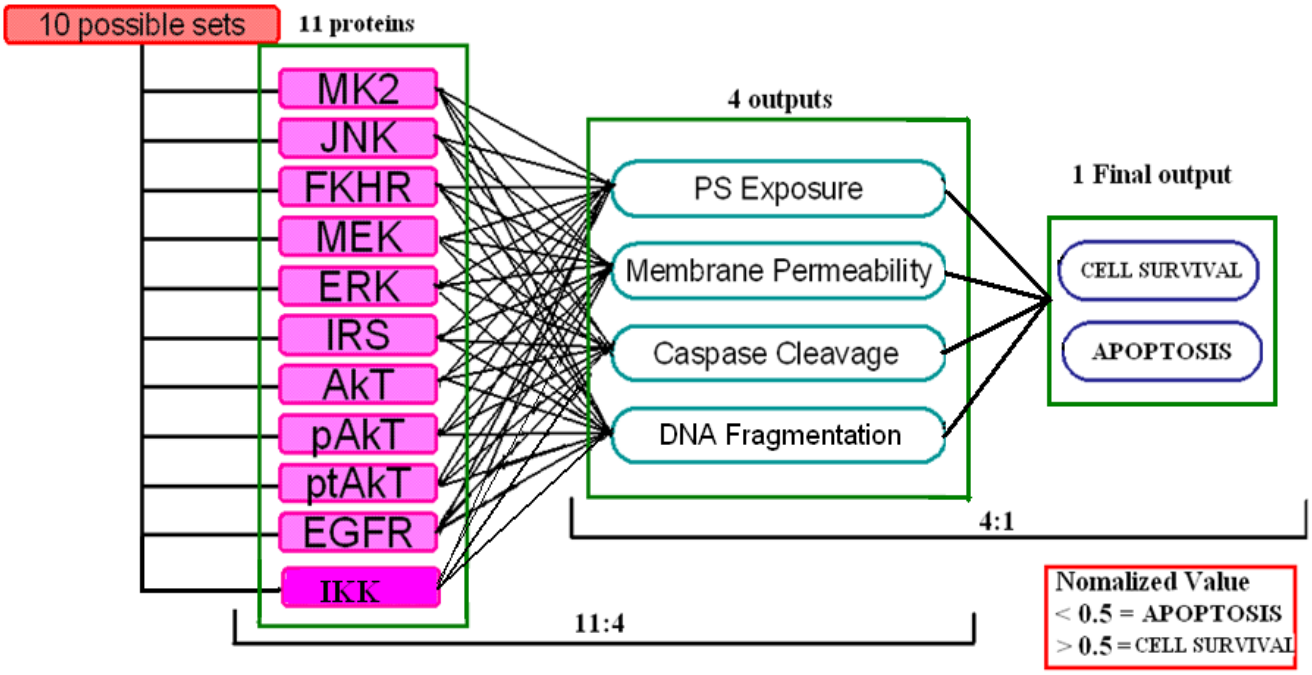


Fig 3.1 Architecture of Neural Network Model (MLP)

However, prediction performances of the first layer of ANN model (11:4) was not enough to estimate because the training data might only include a relatively small number of data points against the number of variables. For prediction performance of the ANN

model, second layer of ANN model (4:1) was built using the synthesized training data. The training perfection and test perfection of 10 possible combinations of 11: 4 and 4:1 data are given in Table 3.1 and Table 3.2.

The experimental data and predicted output from our simulator using artificial neural network is included in Table 3.3.The small differences between the predicted score and experimental score for cell survival/ cell death reveals good predictivity of ANN model.

Table 3.1 The output for every possible set using 11: 4

S. No	Possible Values	Network Name	Training perfection	Test perfection
1	0-0-0	MLP 11-14-4	99.14 %	98.94%
2	5-0-0	MLP 11-8-4	99.76 %	99.67 %
3	100-0-0	MLP 11-5-4	99.77 %	99.76 %
4	0-100-0	MLP 11-15-4	99.20 %	99.22 %
5	5-1-0	MLP 11-15-4	99.58 %	99.30 %
6	100-100-0	MLP 11-16-4	98.48 %	98.43 %
7	0-0-500	MLP 11-15-4	94.47 %	90.58 %
8	0.2-0-1	MLP 11-4-4	99.70 %	99.67 %
9	5-0-5	MLP 11-6-4	99.89 %	99.86 %
10	100-0-500	MLP 11-7-4	98.33 %	98.12

Table 3.2 The final output for every possible set using 4: 1

S. No	Possible Values	Network Name	Training perfection	Test perfection
1	0-0-0	MLP 4-10-1	99.55 %	99.54 %
2	5-0-0	MLP 4-7-1	99.79 %	99.81 %
3	100-0-0	MLP 4-14-1	99.12 %	99.40 %
4	0-100-0	MLP 4-10-1	98.72 %	98.62 %
5	5-1-0	MLP 4-3-1	99.91 %	99.90 %
6	100-100-0	MLP 4-14-1	99.85 %	99.85 %
7	0-0-500	MLP 4-5-1	99.16 %	97.83 %
8	0.2-0-1	MLP 4-15-1	99.08 %	98.65 %
9	5-0-5	MLP 4-9-1	99.93 %	99.93 %
10	100-0-500	MLP 4-13-1	99.94 %	99.93

Table 3.3 Experimental output and its comparison with predicted output using ANN.

S.No	TNF-EGF-Insulin Treatments	Experimental output		Predicted output using our simulator	
		Score	**Categorical**	**Score**	**Categorical**
1	100-100-0	0.559	Cell Survival	0.552	Cell Survival
2	0.2-0-1	0.605	Cell Survival	0.608	Cell Survival
3	5-0-5	0.553	Cell Survival	0.551	Cell Survival
4	100-0-500	0.359	Cell death	0.352	Cell death

3.2 SYSTEM IMPLEMENTATION OF DETERMINISTIC MODELING : There is a balance between cell death and survival in living organisms. The ability to sense their environment and decide to survive or die is dependent largely upon growth factors. Studies of signaling pathways have traditionally focused on delineating immediate upstream and down stream interactions, and then organizing these interactions into linear cascades that relay and regulate information from cell surface receptors to cellular effectors such as metabolic enzymes, channels or transcription factors [79]. This work examines signaling networks that control the survival/ death decision treated with combinations of three primary signals the pro death cytokine, *tumor necrosis factor-α* (TNF) and the pro survival growth factors, *epidermal growth factor* (EGF) and insulin.

3.2.1 TNF α SIGNAL CASCADE

Modeling TNF activity on cells

TNF is a homo trimeric molecule that binds to two different receptors: TNF-R1 and TNF-R2. TNF-R1 appears to be the key mediator of TNF signaling in both normal and tumor cells and for this reason we focus our analysis on this receptor. TNF-R1 has three subunits, and their cytoplasmic tails must be juxtaposed to trigger intracellular signaling. Two models of TNF-R1 subunits recruitment have been proposed over the years: the first assumes that the receptor subunits juxtapose upon binding of homo trimeric TNF which, therefore, would drive the assembly of active TNF-R1; the second, which is supported by recent experimental evidences, suggests that TNF-R1 subunits self-assemble in the

absence of TNF thanks to a conserved extra cellular domain called the Pre-Ligand Assembly Domain (PLAD). In this case, signaling by pre-assembled receptors before TNF binding would be prevented by cytosolic negative regulators such as the Silencer of Death Domain (SODD). Binding of TNF to TNF-R1 initiates a series of biochemical events in the cell that take place at the cytoplasmic tails of the receptor subunits and, in particular, at their specialized domains called Death Domains (DD). DD recruit the adaptor protein TRADD that acts as an assembly platform for at least two other proteins, RIP-1 and TRAF-2. This multi protein complex initiates the signaling cascades resulting in NF-κB activation and JNK activation and hence gene activation and cell survival. Among the genes that are expressed after NF-κB activation and JNK activation, there are those that code for the two proteins FLIP and IAP that inhibit the TNF apoptotic pathway [80, 81].

It has been recently demonstrated that the TNF apoptotic pathway is initiated by TNF/TNF-R1 complexes internalized into endocytic vesicles. At this intracellular level, the multi protein complexes associated to the receptors' tails modify and form the so-called Death Inducing Signaling Complex (DISC), whereby TRADD recruits FADD and pro-caspase-8. This caspase then triggers the irreversible pathway leading to cell death. It has also been demonstrated that the fate of endosomes containing TNF/TNF-R1 complexes prior to their maturation into lysosomes is to fuse with vesicles from the trans-Golgi network. The NF-κB, JNK and the apoptotic pathways comprise a series of complex intracellular reactions involving a number of enzymes and substrates. These have been the subject of intense modeling efforts aimed at explaining the response of individual cells to TNF from a systemic perspective at the molecular level.

3.2.1.1 Binding and internalization of TNF/TFN-R1 complexes

The model by Bajzer *et al*. (1989) fitted experimental data well; we use it to describe the early events of TNF interactions with cells. However the original model of Bajzer *et al.* (1989) must be updated to account for some novel aspects of TNF biology. In particular, Bajzer *et al.* (1989) assumed that internalized ligand/receptor complexes could be recycled back at the cell surface. It is highly probable that TNF/TNF-R1 complexes do

not recycle at all but are finally degraded into lysosomes. Therefore we modify the model by Bajzer *et al* (1989) and Chignola *et al* (2009) model as follows

$$\frac{d[R]}{dt} = V_r - k_d[R] - k_{on}[L][R] + k_{off}[N_c]$$
$$\frac{d[L]}{dt} = -k_{on}[L][R] + k_{off}[N_c]$$
$$\frac{d[N_c]}{dt} = k_{on}[L][R] - (k_{off} + k_{in})[N_c]$$
$$\frac{d[N_{in}]}{dt} = k_{in}[N_c] - k_{\deg}[N_c]$$

...(3. 1)

where square brackets denote molar concentrations of free TNF-R1 receptors (R), free TNF (L), TNF/TNF-R1 complexes bound at the cell membrane (N_C) and internalized complexes (N_{in}). Here k_{on} and k_{off} are the association and dissociation rate constants for TNF binding to TNF-R1, respectively, k_{in} is the internalization rate constant of TNF/TNF-R1 complexes and k_{deg} is the rate constant of lysosomal degradation of the complexes.

The two parameters V_r and k_d were introduced by Bajzer *et al* 1989 although with a slightly different notation, to describe "the zero-order rate of insertion of receptors into the membrane and the turnover (internalization) rate constant of ligand-free receptors" [79, 80] respectively.

3.2.1.2 Modeling the intracellular signaling pathways triggered by TNF

Fig.3.2 shows how our minimal model maps onto the main biochemical paths triggered by TNF binding to its receptors that demonstrates the pathway leading to NF-κB activation, JNK activation and cell survival is initiated at the cells surface upon the formation of TNF/TNF-R1 complexes, whereas the one that leads to cell death and cell death by internalized complexes. In addition to the basic observations by Schneider-Brachet et al., we have integrated these circuits by implementing the NF-κB and JNK-mediated transcription of genes coding for caspase-8 inhibitors such as FLIP. In this way,

the three intracellular pathways interact dynamically, in as much as the cell survival pathway - that starts earlier since it does not require internalization of TNF/TNF-R1 complexes - can inhibit the apoptotic path. Here we model both biochemical circuits by means of three molecular species, that we denote with C, *B* and *A*, that collectively summarize the various reactions leading to cell survival and death, respectively, and the interplay between the three paths. The, molecules *C*, B and *A* can be loosely identified with JNK/FLIP, NF-κB/FLIP and caspase-8, respectively (Fig. 3.1). We assume that after the initial trigger pathways proceed irreversibly to their endpoint. In this way we neglect many details of pathways which involve a number of different molecular actors, and thus we neglect all those reactions that probably serve to fine tune the effects of TNF. The equations for *A, B,* and *C* are:

$$\frac{d[B]}{dt} = \beta[N_c] - k_{B\deg}[B]$$
$$\frac{d[C]}{dt} = \delta[N_c] - k_{C\deg}[C]$$

...(3. 2)

where the variables N_C and N_{in} are the same as in the differential system shown in Eq 3.1. We see from the differential system Eq 3.2, that the cell survival signal, modeled phenomenologically by means of the chemical species *B* and *C*, depends on the number of TNF-TNF-R1 complexes at the cell surface (e.g. N_C), with rate constant parameter β and δ. Finally, C and *B* can be degraded by means of ubiquitination and proteasome cleavage and/or irreversibly inhibited by other molecular species, and these processes are described by the rate constants k_{Cdeg} and k_{Bdeg}, respectively.

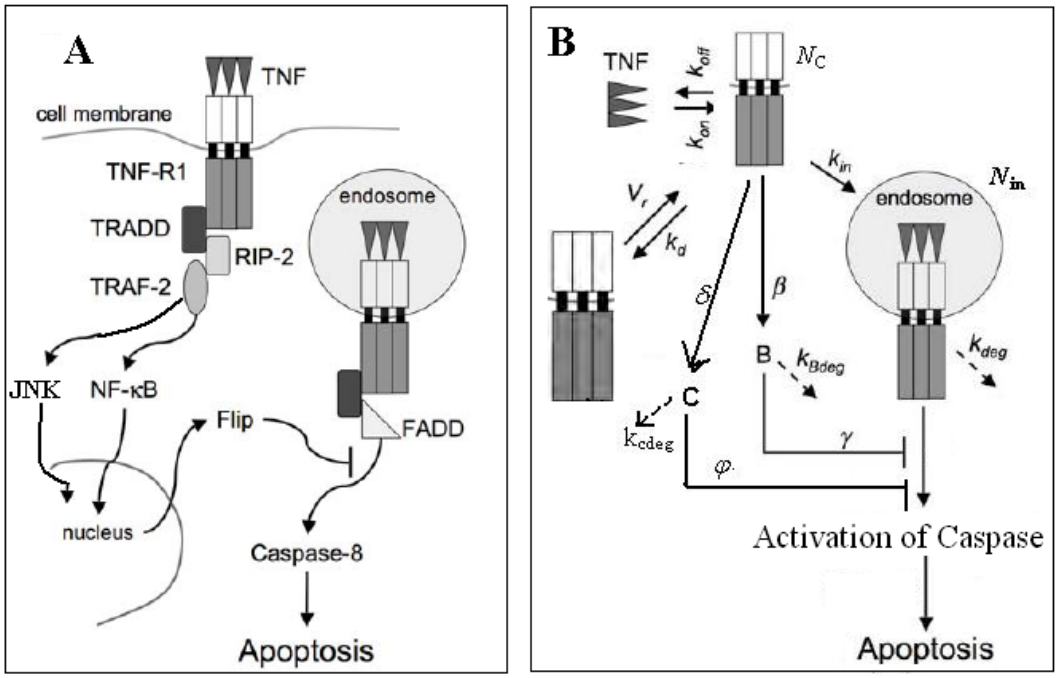


Fig 3.2 Modeling TNF cytotoxicity. **A.** Biological view of the main TNF paths. **B.** Scheme of the biochemical paths that have been considered in the present model.

3.2.2 EGF SIGNAL CASCADE

Modeling EGF activity on cells

The epidermal growth factor (EGF) and EGF receptor (EGFR) were among the first growth factor ligand-receptor pairs discovered. The epidermal growth factor receptor (EGFR) family plays an important role in cell lineage determination, the morphogenesis of many organs and in cell survival in the adult. Moreover, activating mutants and over-expression of these family members contribute to oncogenesis by inducing cells to proliferate and to resist cell death. Subsequent phosphorylation of the EGF itself and/or other proteins, which then pass on to various signaling cascades [e.g., phosphatidylinositol 3-kinase (PI3K)/Akt, JAK/STAT and Ras/ ERK pathway], can lead to different cellular events such as growth, migration, and division.

Upon ligand-binding receptors homo-dimerise or hetero-dimerise triggering tyrosine trans-phosphorylation of the receptor sub-units. Intracellular tyrosine kinases of the Src family and Abl family are also able to tyrosine phosphorylate ErbB receptors. These tyrosine phosphorylated sites allow proteins to bind through their Src homology 2 (SH2) domains leading to the activation of downstream signaling cascades including the

RAS/extracellular signal regulated kinase (ERK) pathway, the phosphatidylinositol 3 kinase(PI3K)/ AkT pathway. Intermediate transcription factors involved in this process are NFκB and JNK. The activation of phosphatidylinositol 3 (PI3) kinase by extracellular growth factors induces phosphorylation, nuclear export, and transcriptional inactivation of FKHR1, a member of the FKHR subclass of the forkhead family of transcription factors. Protein kinase B (PKB)/Akt, a key mediator of PI3 kinase signal transduction, phosphorylated recombinant FKHR1 These results indicate that phosphorylation by PKB/Akt negatively regulates FKHR1 by promoting export from the nucleus.

EGF activates the ERK pathway through the binding of Grb2 or Shc to phosphorylated ErbB receptors, which in turn results in the recruitment of the son of sevenless (SOS) to the activated receptor dimmer SOS then activates RAS leading to the activation of RAF 1. RAF-1 subsequently phosphorylates MEK1 and MEK2 which activate ERK1 and ERK2 respectively. This pathway results in cell proliferation and in the increased transcription of Bcl2 family members and inhibitors of cell death proteins (IAPs), thereby promoting cell survival. The ERK pathway also responds to mitogen activation which leads the activation of JNK/SAPK pathway SAPK stands for stress activation protein kinases and within this class of kinases the Jun N-terminal kinases (JNK) for a subfamily.

Another signaling cascade initiated by EGF is the JAK/STAT pathway, which is also implicated in cell survival responses. JAK phosphorylates STAT proteins localized at the plasma membrane. This leads to the translocation of STAT proteins to the nucleus where they activate the transcription of genes associated with cell survival.

3.2.2.1 Modeling the intracellular signaling pathways triggered by EGF

Fig.3.3 shows how our minimal model maps onto the main biochemical paths triggered by EGF binding to its receptors. The figure, i.e. our modeling effort, demonstrates that the pathway leading to NF-κB activation, JNK activation, MEK/ERK activation, JAK/ STAT activation and cell survival is initiated at the cells surface upon the formation of EGF/EGFR complexes, whereas the one that leads to cell death by internalized complexes. We have integrated the circuits by implementing the NF-κB and JNK-

mediated transcription of genes coding for caspase-3 inhibitors such as FLIP. In this way, the intracellular pathways interact dynamically, in as much as the cell survival pathway - that starts earlier since it does not require internalization of EGF/EGFR complexes - can inhibit the apoptotic path [81]. Here we model biochemical circuits by means of six molecular species, that we denote with *G*, *F*, *E*, C, and *B* that collectively summarize the various reactions leading to cell survival and death respectively, and the interplay between the six paths. The, molecules *G*, *F*, *E*, *C*, and B can be loosely identified with JAK/ STAT, FKHR, MEK/ERK, JNK/FLIP and NF-κB/FLIP respectively (Fig.3.2). We assume that after the initial trigger pathways proceed irreversibly to their endpoint. The equations for *B, C*, *E F* and *G* are:

$$\frac{d[B]}{dt} = \beta[N_c] - k_{B\deg}[B]$$
$$\frac{d[C]}{dt} = \delta[N_c] - k_{C\deg}[C]$$
$$\frac{d[E]}{dt} = \eta[N_c] - k_{E\deg}[E]$$
$$\frac{d[F]}{dt} = \mu[N_c] - k_{F\deg}[F]$$
$$\frac{d[G]}{dt} = \chi[N_c] - k_{G\deg}[G]$$

...(3. 3)

where the variables N_C and N_{in} are the same as in the differential system (Eq. 3.1). We see from the differential system (Eq. 3.3), that the cell survival signal, modeled phenomenologically by means of the chemical species *B*, *C*, *E* and *G* depends on the number of EGF/EGFR complexes at the cell surface (e.g. N_C), with rate constant parameter β, δ, η, and χ. On the other hand, the apoptotic signal, modeled phenomenologically by means of the chemical species *F* that denotes for FKHR pathway with rate constant parameter μ, depends on the number of internalized ligand/receptor complexes (e.g. N_{in}) with rate constant α. Finally, *G*, *F*, E, C and *B* can be degraded by means of ubiquitination and proteasome cleavage and/or irreversibly inhibited by other molecular species, and these processes are described by the rate constants k_{Gdeg}, k_{Fdeg}, k_{Edeg} , k_{Cdeg} and k_{Bdeg}, respectively.

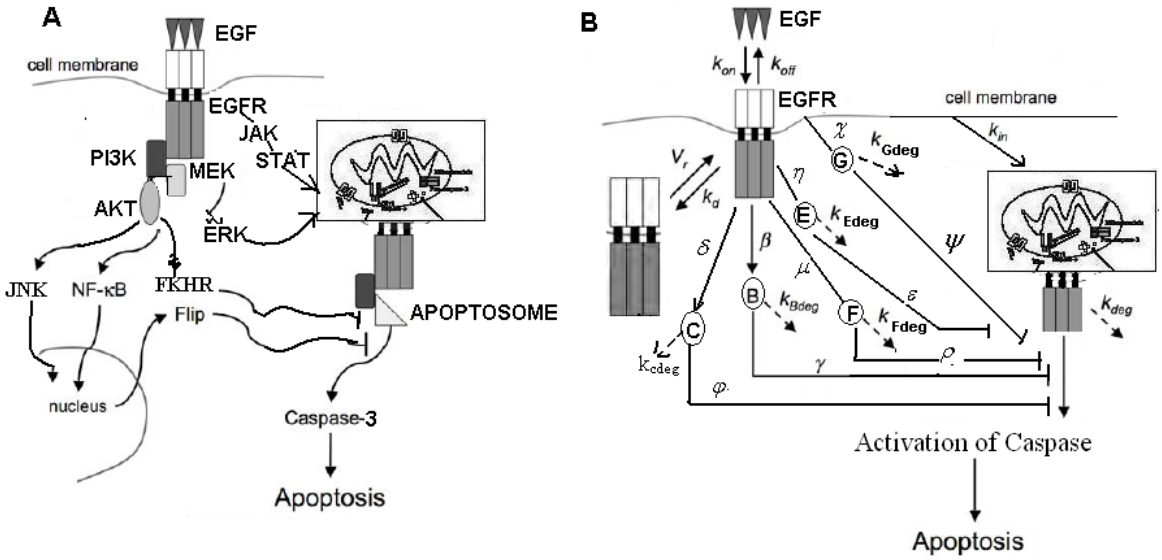


Fig 3.3 Modeling EGF cytotoxicity. **A.** Biological view of the main EGF paths. **B.** Scheme of the biochemical paths that have been considered in the present model.

3.2.3 INSULIN

Modeling INSULIN activity on cells

Insulin is a hormone that regulates the amount of glucose (sugar) in the blood and is required for the body to function normally. It is synthesized as one chain, which is then cleaved at one site by a protease to form a two chain (A and B) protein. The two chains are covalently linked by a bond (disulfide) between a cysteine, an amino acid with a -CH_2SH side chain, with a cysteine side chain on the other chain. This protein binds to its receptor, the insulin receptor, on cell membranes, which initiates a process of signal transduction. The insulin receptor is a hormone-dependent kinase. When insulin binds on the extracellular part of the insulin receptor, shape changes, communicated to the intracellular part, cause it to bind ATP and phosphorylate proteins, specifically on tyrosine side chains. The insulin receptor is an insulin-dependent tyrosine kinase. Insulin binds to the extracellular α-subunit of the receptor and induces a conformational change that brings the α-subunits closer together. This leads to a rapid autophosphorylation of the receptor. This then allows other intracellular proteins to bind to the intracellular domain of the receptor, and become phosphorylated.

PI3K is activated by insulin, insulin-like growth factor-1 and other growth factors. PI3K is a heterodimeric lipid kinase with a broad range of cellular functions, including growth and differentiation, synthesis and degradation of carbohydrates, proteins and lipids, and membrane trafficking. PI3K consists of a regulatory subunit that associates with a catalytic subunit. The regulatory subunit binds the IRSs, whereas the catalytic subunit phosphorylates in the membrane. PDK (phosphoinositide-dependent protein kinase)/Akt (protein kinase B, c-Akt) is one of the serine/threonine kinases downstream of PI3K. Intermediate transcription factors involved in this process are NFκB and JNK.

Using cell biological, biochemical, genomic, and proteomic approaches, we have uncovered the complex molecular understanding of a signaling network centered around a G protein switch involving the tuberous sclerosis complex (TSC) tumor suppressors (TSC1 and TSC2) and the Ras-related small G protein Rheb. A complex between TSC1 and TSC2 is regulated by multi-site phosphorylation and acts as a point of integration for a diverse array of cellular signals, including those arising from growth factors, nutrients, and a variety of stress conditions. When active, the TSC1-TSC2 complex [36, 37] acts as a GTPase activating protein (GAP) for Rheb, thereby turning Rheb off by stimulating its intrinsic GTPase activity. In the presence of growth factors and nutrients, this complex is turned off, allowing the GTP-bound active version of Rheb to accumulate and turn on downstream pathways. The best-characterized downstream effectors of Rheb is the mammalian target of rapamycin complex 1 (mTORC1), a critical regulator of cell growth and cell proliferation.

Although genetic analysis has demonstrated that members of the winged helix, or forkhead, family of transcription factors play pivotal roles in the regulation of cellular differentiation and proliferation, little is known of the mechanisms underlying their regulation. Here we show that the activation of phosphatidylinositol 3 (PI3) kinase by extracellular growth factors induces phosphorylation, nuclear export, and transcriptional inactivation of FKHR1, a member of the FKHR subclass of the forkhead family of transcription factors. Protein kinase B (PKB)/Akt, a key mediator of PI3 kinase signal transduction, phosphorylated recombinant FKHR1. These results indicate that

phosphorylation by PKB/Akt negatively regulates FKHR1 by promoting export from the nucleus.

Another signaling cascade initiated by Insulin is the JAK/STAT pathway, which is also implicated in cell survival responses. JAK phosphorylates STAT proteins localized at the plasma membrane. This leads to the translocation of STAT proteins to the nucleus where they activate the transcription of genes associated with cell survival.

3.2.3.1. Modeling the intracellular signaling pathways triggered by INSULIN

Fig.3.4 shows how our minimal model maps onto the main biochemical paths triggered by Insulin binding to its receptors. The figure demonstrates seven molecular species, that we denote with *H*, *G*, *F*, *E*, *C* and *B*, that collectively summarize the various reactions leading to cell survival and death, respectively, and the interplay between the seven paths. The, molecules *H*, *G*, *F*, *E*, *C* and *B* can be loosely identified with mTOR, JAK/STAT, FKHR, MEK/ERK, JNK/FLIP and NF-κB/FLIP respectively (Fig.3.4). We assume that after the initial trigger both pathways proceed irreversibly to their endpoint. In this way we neglect many details of both pathways which involve a number of different molecular actors, and thus we neglect all those reactions that probably serve to fine tune the effects of Insulin. The equations for *B, C*, *E*, *F*, *G* and *H* are:

$$\frac{d[B]}{dt} = \beta[N_c] - k_{B\deg}[B]$$
$$\frac{d[C]}{dt} = \delta[N_c] - k_{C\deg}[C]$$
$$\frac{d[E]}{dt} = \eta[N_c] - k_{E\deg}[E]$$
$$\frac{d[F]}{dt} = \mu[N_c] - k_{F\deg}[F]$$
$$\frac{d[G]}{dt} = \chi[N_c] - k_{G\deg}[G]$$
$$\frac{d[H]}{dt} = \lambda[N_c] - k_{H\deg}[H]$$

...(3. 4)

We see from the differential system (Eq. 3.4), that the cell survival signal, modeled phenomenologically by means of the chemical species *B*, *C*, *E* and *H* depends on the

number of Insulin/ IRS complexes at the cell surface (e.g. N_C), with rate constant parameter β, δ, η, χ and λ. On the other hand, the apoptotic signal, modeled phenomenologically by means of the chemical species *F*, that denotes FKHR pathway, with rate constant parameter μ respectively. Finally, H, G, F, E, C and *B* can be degraded by means of ubiquitination and proteasome cleavage and/or irreversibly inhibited by other molecular species, and these processes are described by the rate constants k_{Hdeg} ,k_{Gdeg}, k_{Fdeg}, k_{Edeg}, k_{Cdeg} and k_{Bdeg} respectively.

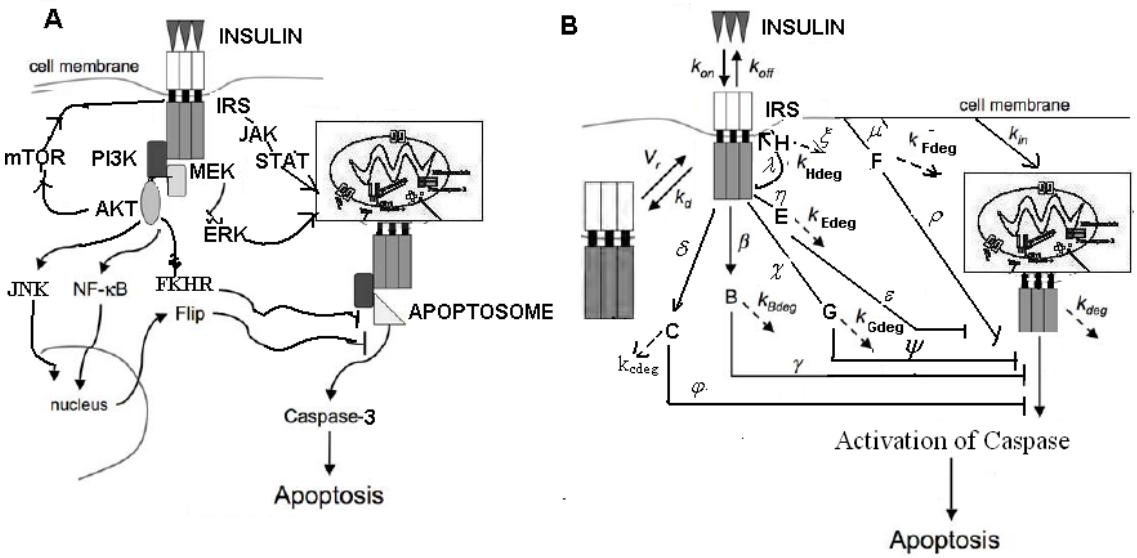


Fig 3.4 Modeling Insulin cytotoxicity. **A.** Biological view of the main Insulin paths. **B.** Scheme of the biochemical paths that have been considered in the present model.

We have developed a minimal quantitative model of TNF, EGF and Insulin cytotoxicity. The model is minimal because it takes into consideration only those reactions that, in our opinion, are essential to describe the action of TNF, EGF and Insulin on cell survival and cell death. We have modeled TNF, EGF and Insulin binding and its uptake by cells shown in Fig 3.5, and it is worth noting that the estimated parameter values are biologically relevant.

The approximation of cell signaling induced by TNF, EGF and insulin leading to cell survival/ cell death model used for mathematical implementation is shown in Figure 14. We define six molecular species, *B*, *C*, *E, F, G* and *H* that collectively summarize the various reactions leading to cell survival/ cell death. The molecules *B*, *C*, *E, F, G* and *H* can be loosely identified with NF-κB/FLIP, JNK/FLIP, MEK/ERK, FKHR, JAK/STAT and mTOR respectively. We assume that after the initial triggering of the pathway, it

precedes irreversibly to its endpoint -- the equations for *B, C*, *E*, *F*, *G* and *H* can be written as:

$$
\begin{aligned}
\frac{d[B]}{dt} &= \beta[N_c] - k_{B\deg}[B] \\
\frac{d[C]}{dt} &= \delta[N_c] - k_{C\deg}[C] \\
\frac{d[E]}{dt} &= \eta[N_c] - k_{E\deg}[E] \\
\frac{d[F]}{dt} &= \mu[N_c] - k_{F\deg}[F] \quad (Death) \\
\frac{d[G]}{dt} &= \chi[N_c] - k_{G\deg}[G] \\
\frac{d[H]}{dt} &= \lambda[N_c] - k_{H\deg}[H]
\end{aligned}
$$

...(3. 5)

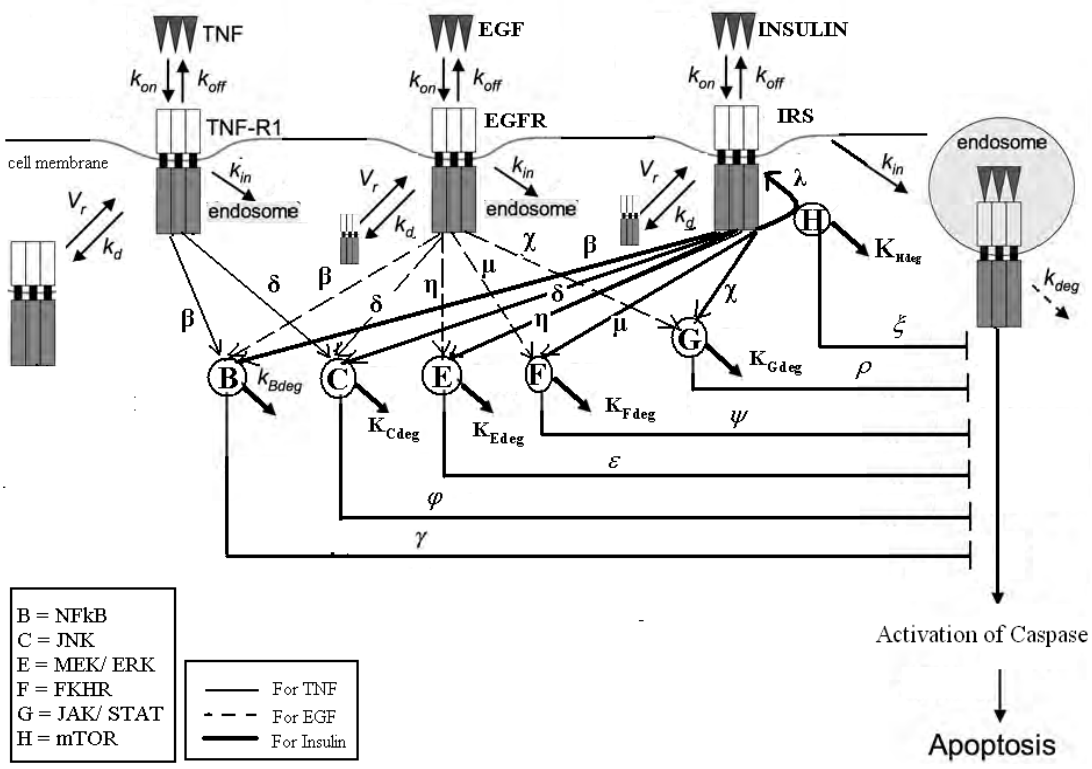


Fig 3.5 Schematic representation of the minimal system model triggered by TNF, EGF and insulin regulating cell survival/ cell death.

We see from the differential system (Eq. 3.5), that the cell survival signal, modeled phenomenologically by means of the chemical species *B*, *C*, *E, G* and *H* depends on the

rate constant parametersβ, δ, η, χ and λ. On the other hand, the apoptotic signal, modeled phenomenologically by means of the chemical species *F* that denotes FKHR pathway, with a rate constant μ. Finally, *B, C*, *E*, *F*, *G* and *H* can be degraded by means of ubiquitination and proteasome cleavage and/or irreversibly inhibited by other molecular species, and these processes are described by the rate constants k_{Bdeg}, k_{Cdeg} ,k_{Edeg}, k_{Fdeg}, k_{Gdeg} and k_{Hdeg} respectively.

Numerical Methods

Let us consider a differential equation as

$$\frac{dy}{dx} + Py = Q \qquad \text{...(3. 6)}$$

$\frac{d}{dx}$ can be written as D, replacing in Eq 3.6 we get

$$Dy + Py = Q \qquad \text{...(3. 7)}$$

Now, we know the $\boxed{\text{Integarting factor} = e^{\int P dx}}$

Multiply Eq.3.7 with Integrating factor both sides, we get

$$e^{\int P dx}.(Dy + Py) = e^{\int P dx}.Q$$

$$Dy e^{\int P dx} + Py e^{\int P dx} = e^{\int P dx}.Q$$

$$Dy e^{\int P dx} = e^{\int P dx}.Q$$

Integrating Both Sides

$$y e^{\int P dx} = \int Q.e^{\int P dx} dx + c$$

Finally the solution of equation is

$$\boxed{\mathbf{y} = \frac{\int \mathbf{Q.e^{\int P dx}\, dx + c}}{\mathbf{e^{\int P dx}}}} \qquad \text{...(3. 8)}$$

Now our main equation is $\frac{d[B]}{dt} + k_{B\text{deg}}[B] = \beta[N_c]$

...(3. 9)

If we compare the above equation i.e Eq. 3.9 with Eq. 3.7 we get

$$y = B ; P = k_{B\deg} \; ; \; Q = \beta [N_c]$$

Putting all these values in Eq. 6 , we get

$$\boxed{B = \frac{\int \beta N_c . e^{\int k_{B\deg} dt} \, dt + c}{e^{\int k_{B\deg} dt}}}$$

...(3. 10)

$$B = e^{-k_{B\deg} t} \left[\left(\int \beta N_c . e^{k_{B\deg} t} \, dt \right) + c \right]$$

After solving the above equation we get

$$B = \frac{\beta N_c}{k_{B\deg}} + c . e^{-k_{B\deg} t}$$

...(3. 11)

Now applying the initial condition i.e. $t = 0$ and $B = 0$; in the Eq. 3.11, we get

$$c = -\frac{\beta N_c}{k_{B\deg}}$$

Now putting the value of c in Eq 3.10, we get

$$\boxed{\mathbf{B = \frac{\beta N_c}{k_{B\deg}} \left(1 - e^{-k_{B\deg} t} \right)}}$$

...(3. 12)

Similarly we can solve all the equations.

3.2.4 RESULTS AND DISCUSSION

3.2.4.1 Differential equation modeling activity of JNK

We have solved the differential equation of JNK as

$$\frac{d[C]}{dt} + k_{C\deg} [C] = \delta [N_c]$$

Solution comes out to be

$$[C] = \frac{\delta [N_c]}{k_{C\deg}} \left[1 - e^{-k_{C\deg} t} \right]$$

...(3. 13)

By assuming the values of $\delta[N_C]$ & $k_{C\deg}$ from Bajzer *et al* (1989) and Chignola *et al* (2009) and considering the different values of time from Gaudet *et al* (2005) we have calculated the corresponding values of C. Figure 3.6 shows the theoretical and experimental results of JNK.

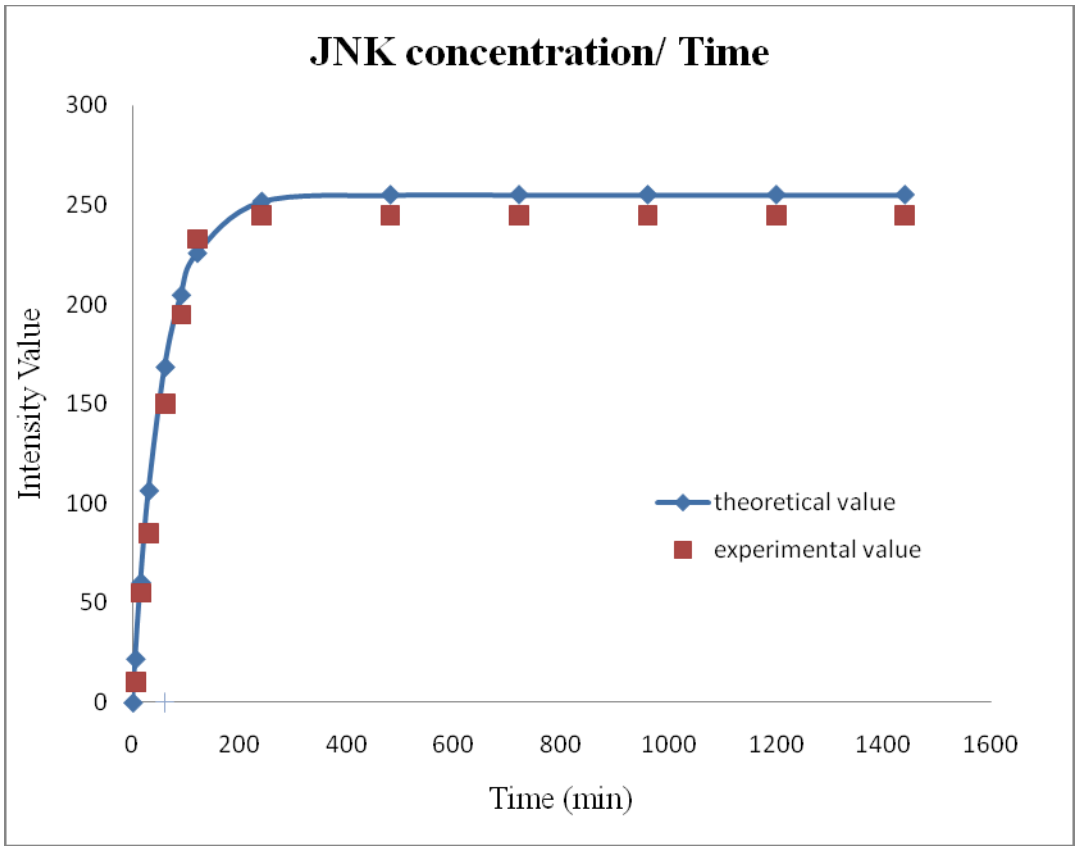


Fig 3.6 : Scatter plot of experimental and theoretical values of JNK. The Root mean square (RMS) error is in the range of 5.33 to 21.39

3.2.4.2.Differential equation modeling activity of MEK/ERK

We have solved the differential equation of MEK/ ERKas

$$\frac{d[E]}{dt}+k_{E\deg}[E]=\eta[N_c]$$

Solution comes out to be

$$[E]=\frac{\eta[N_c]}{k_{E\deg}}\left[1-e^{-k_{E\deg}t}\right]$$

...(3. 14)

By assuming the values of values of $\eta[N_C]$& $k_{E\deg}$ from Bajzer *et al* (1989) and Chignola *et al*(2009) and taking different values of time from Gaudet *et al* (2005) we have calculated the values of E. Figure 3.7 shows the theoretical and experimental results of MEK.

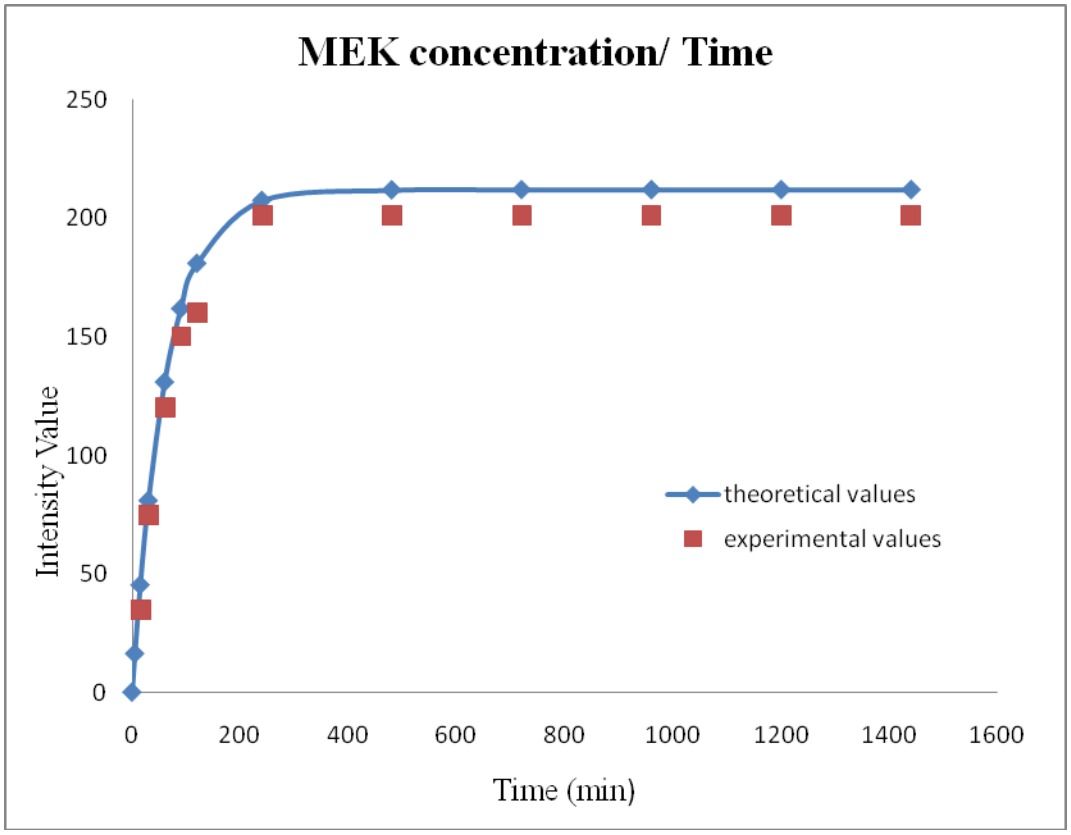


Fig 3.7 : Scatter plot of experimental and theoretical values of MEK. The Root mean square (RMS) error is in the range of 0 to 21.9.

3.2.4 3. Differential equation modeling activity of FKHR

We have solved the differential equation of FKHR as

$$\frac{d[F]}{dt}+k_{F\deg}[F]=\mu[N_c]$$

Solution comes out to be

$$[F]=\frac{\mu[N_c]}{k_{F\deg}}\left(1-e^{-k_{F\deg}t}\right)$$

...(3. 15)

By assuming the values of values of $\mu[N_C]$& $k_{F\deg}$ from Bajzer *et al* (1989) and Chignola *et al*(2009) and by taking the different values of time from Gaudet *et al* (2005) we have calculated the values of F. Figure 3.8 shows the theoretical and experimental results of FKHR.

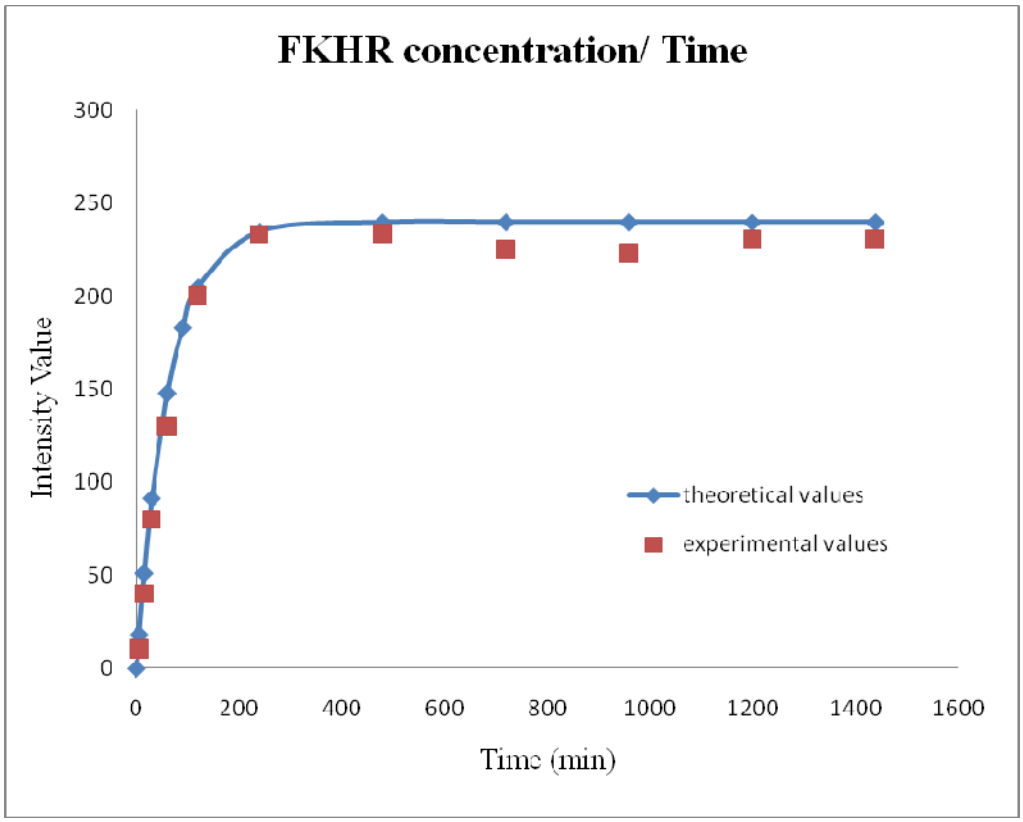


Fig 3.8 : Scatter plot of experimental and theoretical values of FKHR. The Root mean square (RMS) error is in the range of 1.62 to 18.1.

We have presented an integrated theoretical framework for describing the balance between cell survival/ cell death regulated by TNF, EGF and insulin. The theoretical models consider here for mathematical intervention is minimal in the sense it takes into consideration only those reactions that are essential to describe the action of TNF, EGF and Insulin on cell survival/ cell death. We have modeled TNF, EGF and Insulin binding and its uptake by cells, and it is worth noting that the essential parameter values are biologically relevant. The results illustrated that the rate constants defined in the deterministic models are biologically relevant of key proteins in the signaling pathway which decide the fate of cell. The quality of fit between theoretical and experimental

values has been represented. The minimum root mean square error (RMSE value) between theoretical and experimental values for the marker proteins revealed good accuracy of the model.

CONCLUSION

Cells respond to stress in a variety of ways ranging from activation of pathways that promote survival to eliciting programmed cell death that eliminates damaged cells. The data in this paper show that cytokine-signal-response compendia should be constructed using measurements that are well distributed across a signaling network, although sparse coverage of the network is acceptable. Nonetheless, re sampling a subset of nodes using multiple assays helps to verify the consistency of heterogeneous data. Experimental validation of measurements is best carried out under conditions in which all signals have a sufficiently large dynamic range for correlation coefficients to be meaningful. Our work illustrates how a complex signaling network can be reduced empirically to a much simpler computational model that is directly tied to biological mechanism.

In a multi-cellular organism, cells constantly receive signals on their internal condition and surrounding environment. In response to various signals, cells proliferate, move around or even undergo suicide. The signal-response is controlled by complex molecular machinery, understanding of which is an important goal of basic molecular biological research. Such understanding is also valuable for clinical application, since lethal diseases like cancer show maladaptive responses to growth-regulating signals. Because the multiple feedbacks in the molecular regulatory machinery obscure cause-effect relations, it is hard to understand these control systems by intuition alone. Here we translate the molecular interactions into differential equations and recapture the cellular physiological properties with the help of numerical simulations and non-linear dynamical tools. The models address the physiological features of programmed cell death, the cell fate decision.

It has been revealed that survival and cell death signals induced by TNF, EGF and insulin are temporarily separated and this is reflected in our model by the differences between the values of the parameters used. Simulations based on electronic implementation (VHDL, SPICE, Fuzzy) and mathematical modelling (non-linear modelling and deterministic model) recapitulate most features of the data and generate several predictions involving pathway crosstalk and regulation. We uncover a

relationship between the key proteins involved in TNF, EGF and insulin cellular signalling pathways that might account for the cell survival and cell death decision of the cells. More generally, these models are flexible, able to incorporate qualitative and noisy data, and powerful enough to produce quantitative predictions and new biological insights about the operation of signalling networks.

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