TETRASPANIN CD9 REGULATES APOPTOSIS IN MOUSE EMBRYONIC STEM CELLS THROUGH THE SUPPRESSION OF EPIDERMAL GROWTH FACTOR RECEPTOR SIGNALING

by

RODNEY JAMES NASH

(Under the Direction of STEPHEN DALTON)

ABSTRACT

CD9, a member of the tetraspanin superfamily, is expressed in several cell lines and is associated with cellular activities such as migration, proliferation and metastasis in cancer cell lines. Antibody ligation of CD9 has been shown to induce cell death in mouse embryonic stem cells and specific cancer cell lines. Here we have shown CD9 antibody induces cell death in mouse R1 and D3 cells through the release of cytochrome C and cleavage of caspase-3, which are hallmarks of apoptosis. Subsequently, phosphorylation of p38 MAPK, SAPK/JNK and ERK 1/2 occurs as a result of treatment with anti-CD9 antibody (KMC8). Furthermore, we have shown HB-EGF and p38 inhibitor perturbs p38 MAPK phosphorylation, promoting cell survival. Additionally, the ligand for DBA (GalNAc) is present on mouse ES cells but noticeably declines during differentiation. We have shown that DBA ligand can be used to identify cells that are positive for the pluripotent state and early differentiation events. These results indicate that normal function of CD9 suppresses apoptosis and DBA ligand can be used to discriminate pluripotent cells from differentiated cells in a mixed population.

INDEX WORDS: Cluster of differentiation 9, Forssman antigen, *Dolichos biflorus* agglutinin, Mitogen activated protein kinase, Stress activated protein kinase/ C-Jun N terminal kinase, Extracellular signal regulated kinase, Apoptosis, Rescue and Juxtacrine Organization (RAJO).

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DEDICATION

I dedicate this piece of work to all of those who seek truth. Truth is conformity to fact or actuality. Over my ten year crusade in becoming a doctor I have seen many truths. The facts are, when it comes to science the average person knows only that it's hard or difficult. Well, it is, but we as scientist do not make it any easier for the common type to begin to understand. We use a form of verbal communication that is more complex than some traditional languages. We as scientist have a responsibility to make sure our common brothers and sisters understand what we do and how they will benefit from our actions. We as scientist must also listen to the voices within our communities regarding concerns for health and well-being, for their cries and concerns are the direction we as scientist should adjust our bearings to and navigate towards. To perform basic scientific activities for the sake of satisfying ones curiosity can be satisfying. To empower a person with knowledge and to improve their life exceeds the conventions of basic science. To serve the community as a scientist is a great responsibility and blessing from God, which I show gratitude for, by making each day I live a day that I give the very best of me…Rodney Nash.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Embryonic Stem Cells: What are they? Where do they come from? What do they do?

Embryonic stem cells (ESCs, ES cells) are unique cells derived from the inner cell mass of the mammalian blastocyst and provide a novel opportunity to study early development events in mammalian system. During early mammalian blastocyst development embryonic stem cells posses the capacity to differentiate into ever cell type of the adult body (Evans and Kaufman, 1981; Martin GR, 1981). Mouse ES cells (mESCs, mES cells) are able to maintain their pluripotency for a long time in cell culture media containing the cytokine leukemia inhibitory factor (LIF) (Smith *et al*, 1988; Williams *et al*., 1988). ES cell grown *in vivo* and *in vitro* have been shown to sustain their pluripotency by their injection into blastocyst and their contribution in embryonic development of all three germ layers (Bradley *et al.* 1984). Additionally, ESCs form teratomas which contain a range of mature tissues when injected into SCID mice (Evans and Kaufman, 1981; Martin GR, 1981). It has been shown that some mESC lines can form entire viable fetuses and newborns when injected into heat-treated blastocyste or tetraploid embryos (Nagy *et al.*, 1993; Amano *et al*. 2000). In the absence of LIF, *in vitro* mESCs start to differentiate to every possible tissue lineage (Nakano *et al*., 1994). For instance, it has been demonstrated that haematopoietic precursor cells can be generated from ES cells by culturing these cells with stromal cells combined with various cytokines (Nakano *et al*., 1994). Additionally, the existence of a common precursor for primitive erythrocytes and other cells of the haematopoietic lineages can develop within cultured embryoid bodies (Kennedy *et al.*, 1997). mES cells can also be directed in controlled systems to differentiate into glial and neural precursors, which can contribute to brain development after transplantation into rodent embryo brains (Brustle *et al.,* 1997). Furthermore, some adult stem cells can be maintained in a multipotent state in tissue culture for an extended period of time (Pittenger *et al.*, 1999).

Contrary to the afermentioned, *in vitro* culture of some adult stem cells sucha s hematopoietic and neural stem cells have not been successfully established as homogeneous stem cell populations (Petersen and Terada, 2001). It is believed that determining how different types of ES cells can be maintained *in vivo* may supply important information regarding the regulation of stem cells and their self renewal abilities. The future looks very promising with regards to clinical applications of stem cells but it is predicated on our understanding of the molecular mechanisms behind *in vitro* maintenance of ES cells (Petersen and Terada, 2001).

Cell Signaling and Apoptosis

Apoptosis is the regulated dominant form of programmed cell death which can occur during embryonic development and normal tissue turnover (Acehan *et al.*, 2002). This death process may also occur as a cellular response to stress or pathogens (Acehan *et al.*, 2002). It is characterized by membrane shrinkage and blebbing and DNA fragmentation (Srinivasula *et al.* 2001). A family of cysteine proteases called caspases are central regulators of apoptosis and during apoptosis, death signals are transduced by biochemical pathways that activate caspases (Li *et al.,* 1997). All caspases are produced in cells as catalytically inactive zymogens and must undergo proteolytic activation during apoptosis (Srinivasula *et al.* 2001 and Li *et al.,* 1997). Initiator caspases-2, -8, -9. -10, -11, and-12 are closely coupled to pro-apoptotic signaling (Srinivasula *et al.* 2001 and Li *et al.,* 1997). These initiator caspases are characterized by an extended N-terminal prodomain (>90 amino acids) which is believed to be important for its function (Li *et al.,* 1997). Initiator caspases lead to the cleavage and activation of effector caspases, which include caspase-3, -6, and -7, which contain effector caspase contains 20–30 residues in its prodomain sequence (Li *et al.,* 1997). Activation of Fas and TNFR by FasL and

TNF leads to the activation of initiator caspase-8 and -10, while DNA damage leads to the activation of initiator caspase-2 (Earnshaw *et al.*, 1999; Li *et al.,* 1997). BH3-only poteins, which are activated in response to membrane stress, promote the opening of Bax/Bak channels in the mitochondria and cytochrome C is released, promotes the assembly of a caspase-activating cmplex called the apoptosome. Included within the apoptosome is the initiator caspase-9, a caspase critical for intracellular amplification of apoptotic signals (Lakhani *et al.,* 2006). Apoptosomes are responsible for the downstream activation of effector caspase-3 and -7, leading to apoptosis (Lakhani *et al.,* 2006). Caspase may feed back directly to the mitochondria and/or may promote the activation of BH3 only proteins to amplify cytochrome C release and triggering loss of mitochondrial function (Lakhani *et al.,* 2006).

Mitogen-activated protein kinases (MAPKs) are a conserved family of serine/threonine protein kinases that are involved in several cellular functions such as cellular differentiation, proliferation, motility and death (Chen *et al.,* 2002). MAPK signaling cascades are prearranged hierarchically into three-tiered modules: MAPKs are activated through phosphorylation by MAPK-kinases (MAPKKs (MLK)), which are phosphorylated and activated by MAPKK-kinases (MAPKKK) (Chen *et al.,* 2002; Sakiyama *et al*., 2005; Rajasingh *et al,* 2006). MAPKKKs are activated by a family of Rho dependent small GTPases and other protein kinases (Chen *et al.,* 2002; Sakiyama *et al*., 2005; Rajasingh *et al,* 2006).

The MAPK (Erk 1/2) signaling pathway an important role in the regulation of cell proliferation (Pan *et al.* 2006). Stimulation through the use of growth factors such as amphiregulin, epiregulin, TGF-α (transitional frowth factor-α), EGF (epidermal growth factor)

and HB-EGF (heparin binding epidermal-like growth factor) induces a protein kinase cascade that activates Raf, MEK, ERK 1/2, Elk-1 and p90RSK (Chokki *et al.,* 2005; Draper *et al.,* 2003).

The stress-activated protein kinase/Jun-N-terminal kinases (SAPK/JNK) are members of the MAPK family and are activated by a variety of environmental stresses which include radiation, oxidative, osmatic and in some situations by growth factors (Murayama *et al.,* 2004). Stress signals are delivered to the cascade by small GTPases of the Rho family (Hammaker *et al.,* 2004). As with other MAPKs, a signaling cascades exist and is prearranged hierarchically into three-tiered modules:MKKKs (MEKK, MLK), MKKs and MKs (Hammaker *et al.,* 2004). SAPK/JNK signaling translocates to the nucleus where it can regulate the activity of several transcriptional factors that effect proliferation, survival and apoptosis (Hammaker *et al.,* 2004).

To complete this discussion, p38 MAPK is a member of the MAPK family, and similarly like SAPK/JNK, is activated by a variety of environmental stresses which include ionizing radiation, heat shock, oxidative stress and osmotic stress (Du *et al.,* 2006; Chan-Hui *et al.*, 1998). As with other MAPKs, signaling involves MKKKs, MEKKs and MLKs phosphorylating MKKs, specifically MKK 3/6, which in turn activates p38 MAPK (Chan-Hui *et al.*, 1998). Small GTPases of the Rho family deliver stress signaling to this cascade (Chan-Hui *et al.*, 1998). There are four p38 MAPK genes (α,β,δ,γ) that have been identified to date in mammalian cells and though all isoforms (particularly δ and γ) potentially activate ATF-2 (transcrition factor that interacts with several oncoproteins and cellular tumor suppressors), isoforms (α and β) are associated with the activation of Hsp25, Hsp27 and MAPKAPK-2, which are involved with translation (Shin *et al.,* 2005; Fassetta *et al*., 2006; Chan-Hui *et al.*, 1998). Furthermore, p38

MAPK regulates other transcription factor such as Elk-1 and MEF2 (Shin *et al.*, 2005; Fassetta *et al*., 2006).

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CHAPTER 2

THE LECTIN DBA RECOGNIZES GLYCAN EPITOPES ON THE SURFACE OF MURINE EMBRYONIC STEM CELLS: A NEW TOOL FOR CHARACTERIZING PLURIPOTENCY AND EARLY CELL FATE COMMITMENT

Cell surface markers are key tools that are frequently used to characterize and separate different cell populations by non-destructive means. Existing markers used to define murine embryonic stem cells (mESCs) such as stage specific embryonic antigen 1 (SSEA1), Forssman antigen (FA) and CD9 are limiting however because they do not unambiguously define the pluripotent state and are not reliable indicators of differentiation commitment. To identify glycan cell surface markers that would circumvent this problem we screened mESCs with a panel of lectins. For a lectin ligand to have utility we specified that presentation of its cognate glycan epitope must be elevated in ESCs. Second, its presentation on the cell surface must decrease with kinetics that precede currently accepted markers such as CD9, SSEA1 and FA. Together, this would establish the glycan marker as having utility as a reliable mESC marker and as a high resolution readout for early cell fate commitment. Here we show that the lectin Dolichos biflorus agglutinin (DBA), recognizes α-N-acetylgalactosamine (GalNAc) containing cell surface epitopes on mESCs ($CD9^{high}$ $SSEA1^{high}$ AP^{high} DBA^{high}). These glycan epitopes however, decline markedly in cells undergoing the first definable step of differentiation, the transition from ESCs to primitive ectoderm ($CD9^{high}$ $SSEA1^{high}$ AP^{high} DBA^{low}). Loss of GalNAc containing epitopes on mESCs is therefore the earliest cell surface change that can be assigned to cells committed to differentiate and the only marker truly specific for pluripotent mESCs. The lectin DBA is therefore a useful tool to identify, by non-destructive approaches, the integrity of mESC cultures and an early indicator of differentiation commitment and developmental potency.

INTRODUCTION

Mouse embryonic stem cells (mESCs, mES cells) are derived from the inner cell mass (ICM) of blastocyst-stage embryos (Baharvand *et al.,* 2004; Saito *et al.,* 2004; Evans *et al.,* 1981). Mouse embryonic stem cells can proliferate indefinitely in an undifferentiated state in the presence of leukemia inhibitory factor (LIF), or differentiate into all three germ layers upon the removal of this factor (Baharvand *et al.,* 2004; Saito *et al.,* 2004; Evans *et al.,* 1981). Identifying mESC markers of pluripotency include the tetraspanin CD9, transcription factors Oct4, Nanog and Rex1, glycolipids SSEA1 (stage specific embryonic antigen 1) and Forssman antigen (FA), and alkaline phosphatase (AP) (Kucia *et al.,* 2006; Oka *et al., 2002;* Rathjen *et al.,* 1999). Although all of these markers are expressed in ES cells, they are less than accurate predictors of pluripotency; as a result of slow down regulation during differentiation. Some markers are still expressed in cells that are irreversibly differentiated (Kucia *et al.,* 2006; Oka *et al., 2002;* Rathjen *et al.,* 1999). Additionally, mRNAs of transcriptional factors Rex 1 and Gbx 2 are closely associated with the pluripotent state and rapidly down regulated during differentiation (Rathjen *et al.,* 1999), but are limited tool for pluripotency identification for a number of reasons: single cell level quantitation of mRNA is difficult; analysis of mRNA typically involves a relatively large numbers of cells and these methods normally involve cell destruction without the recovery of viable cells. It is therefore essential to identify new markers for murine ESCs that are more effective at identifying the pluripotency and which indicate early commitment to differentiation.

Lectins are often used to identify, characterize and isolate unique cell subpopulations based on the presentation of specific carbohydrate moieties on the cell surface. For example,

Lycopersicon (tomato)esculetum lectin (TL) Ricinus communis agglutinin (RCA), and Concanavalin A (Con A) may be used as markers that are associated with the pluripotent state of hESCs (Venable *et al.,* 2005). In this report we describe Dolichos biflorus agglutinin (DBA), which recognizes GalNAc, as being highly reactive towards murine ESCs. α-N-acetylgalactosamine on the cell surface is rapidly down regulated and becomes absent during differentiation, earlier that of SSEA1, CD9 and FA.

In this study we hypothesis and determined that decreased DBA reactivity coincided with the formation of primitive ectoderm, an SSEA1^{high} Oct4^{high} Fgf5^{high} population that represents the first definable differentiation step involved in germ layer formation from ESCs. These findings establish utility for glycan epitopes in the characterization of pluripotent ESCs and identify a new tool that can be used as a non-destructive ESC marker and a reliable readout for initial differentiation events that have not been possible to dissect with existing markers.

Materials and Methods

Cell Culture

D3 and R1 mESCs (Doetschman *et al.,* 1985; Hadjantonakis *et al.* 1988) were cultured in the absence of feeders on tissue culture grade plasticware pre-coated with 0.1% gelatin-phosphate buffered saline (PBS), as described previously (Rathjen *et al.,* 1999) . mESC culture medium consisted of Dulbecco's Modified Eagle Medium (DMEM, Gibco BRL) supplemented with 10% fetal calf serum (FCS, Commonwealth Serum Laboratories), 1 mM L-glutamine, 0.1

mM 2-mercaptoethanol, and 10^3 U/ml recombinant human LIF (ESGRO, Chemicon International) at 37^{o}C under 10% CO_2. EPL cells were formed and maintained by culturing as described previously (Rathjen *et al.,* 1999; Lake *et al.,* 2000; Stead *et al.,* 2002) in mESC-medium containing 50% conditioned medium (MedII) supplemented with 1 mM L-glutamine and 0.1 mM 2-mercaptoethanol, at a density of 2 x 10^4 cells/cm^2. EPL cells were cultured for 2 days (EPL2) or passaged and cultured for a further 2 days to produce EPL4 cells. MedII conditioned medium was prepared from HepG2 cells (ATCC HB-8065) grown to confluence for 4-5 days in DMEM supplemented with 10% FCS, after being seeded at 5 x 10^4 cells/cm^2. Condition medium was collected, filter sterilized then stored at 4^{o}C for up to 2 weeks. Reversion EPL4 cells was carried out as described (Rathjen *et al.,* 1999; Lake *et al.,* 2000; Stead *et al.,* 2002) by reseeding cells into mESC-medium containing LIF and culturing for 2, 4 or 6 days, passaging every 2 to 3 days.

Lectin binding

Cells were harvested to a single cell suspension by trypsinization then washed and resuspended in 0.1% BSA-PBS. Aliquots of 100 µl containing 10^6 cells were mixed with an equal volume of FITC-lectin diluted in 0.1% BSA / PBS, and incubated for 30 min at 4^{o}C. Lectins were used at a final concentration of 20 µg/ml or 100 µg/ml. Cells were then washed once in 5% FCS-95% DMEM and resuspended in 500 µl of the same medium for analysis on a Beckman-Coulter flow cytometer with WinMDi software.

Attached cells were prepared for lectin binding by culturing in 24 well trays to 50-80% confluence, washed twice with PBS, then fixed in 4% paraformaldehyde for 1 hour. Fixed cells were washed twice with PBS before the addition of lectin solution diluted to a concentration of

20 μg/ml or 100 μg/ml in PBS containing 0.1% Bovine Serum Albumin (BSA). Lectin binding was carried out for 30 min at 4^{o}C, and washed with 0.1% BSA / PBS prior to analysis by fluorescence microscopy.

Transcript analysis

RNA was prepared using Qiagen RNeasy Mini Kits and analyzed using micro fluidic technology.

Results

Changes in surface marker expression during differentiation

This experiment was completed to determine the change, if any, in the expression of CD9, DBA and FA glycosphingolipid in three different cell states: mES cells**,** EPL cells and spontaneously differentiated ES cells. Mouse Embryonic Stem Cells were grown under conditions described in the methods and materials section.

ES cells were grown in complete media and differentiation media. ES cells grown in complete media were harvested after one day of growth (~40-60% confluent). ES cells grown in differentiation media were harvested at days two, four and six. Media for differentiation was changed every two days.

EPL cells were grown in MED2 media, as described in the methods and materials section. Cells were collected at days two and four. At day four cells were reverted back to ES cells by the addition of complete media, at which cells were harvested at days two (Day six) and four (Day eight).

Separately, each group of collected sample cells were stained as described in the methods and materials section. Quantitative analysis of CD9 was assessed through flow cytometry using the Beckman Coulter FC500 flow cytometer.

CD9 expression is high (~10^2-10^3 log) in ESCs (Fig 1(. After the removal of LIF, at day 3-4 the CD9 levels decrease approximately by 2 fold and there is evidence of a mixed species of lesser CD9 positive and more positive cells. CD9 expression returns by day 5-6 to levels comparable to day one ESCs. In EPLs the expression of CD9 never recedes to the levels seen in spontaneously differentiated ES cells. Similarly, the level of DBA ligand and Forssman antigen glycosphingolipid lessens during differentiation. EPL cells do not completely lose the DBA ligand, at day 4, and the expression of the DBA ligand returns during reversion. In spontaneously differentiated cells the receptors for DBA ligand do not return. Neither does the round, dome-like morphology that is common with stem cells. On the contrary EPL cells re-gain their pluripotent ES cell morphology during the reversion period. Currently, SSEA-1, CD9 and FA are accepted surface marker for mESCs **(**Pelton, 2002; Rathjen, 1999). These data indicate SSEA-1 positivity in ES cells at day 6 of spontaneous differentiation when pluripotency is no longer present. SSEA-1 alone can not be used as an identifier of ES cells; transcriptional factors such as Oct4, Nanog and Rex1 must also assist (Oka, 2002). This indicates that DBA may be a more useful tool to monitor self-renewing mESCs and early differentiation events than markers currently used.

DISCUSSION

In this study we have shown CD9 is expressed on mouse ES, EPL and spontaneously differentiated cells and additionally, the ligand for DBA and Forssman antigen glycosphingolipid

are also present. In cells that express detectable levels of CD9, SSEA-1 and FA can be discriminated by the presence of their levels of DBA ligand. These carbohydrate moieties change during differentiation and up to now this type of discrimination by DBA or any other lectins has not been established for mESCs, EPL cells and spontaneously differentiated cells.

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` **CHAPTER 3**

THE ROLE OF CD9 IN THE SUPPRESSION OF APOPTOSIS IN MOUSE EMBRYONIC STEM CELLS

CD9, a member of the tetraspanin super family, is expressed in several cell lines and is associated with cellular activities such as migration, proliferation and metastasis in cancer cell lines. Antibody ligation of CD9 has been shown to induce cell death in mouse embryonic stem cells and specific cancer cell lines. Here we have shown CD9 antibody induces cell death in mouse R1 and D3 cells through the release of cytochrome C and cleavage of caspase-3, which are hallmarks of apoptosis. Subsequently, phosphorylation of p38 MAPK, SAPK/JNK and ERK 1/2 occurs as a result of treatment with anti-CD9 antibody (KMC8). Furthermore, we have shown HB-EGF and p38 inhibitor perturbs p38 MAPK phosphorylation, promoting cell survival. These results indicate that normal function of CD9 suppresses apoptosis.

Introduction

CD9 is a ~24-27kD glycoprotein that belongs to the family of tetraspanins, which have been reported to function in a variety of cell activities including cell adhesion, motility, differentiation, migration and signaling (Cha *et al.*, 2000; Hemler *et al.* 2003). Tetraspanins have two extracellular (EC) loops, a smaller EC loop between trans membrane (TM) regions one and two, a larger EC loop between TM regions three and four, an interconnecting intracellular loop (IC) between TM region two and three and cytoplasmic N- and C- termini (Hemler *et al.* 2003; Maecker *et al.*, 1997; Seigneuret *et al., 2001;* Stipp *et al.,* 2003; Wright *et al*, 1994). The existence of tetraspanins became evident in the early 1990's through gene cloning of cell-surface molecules, some of which were identified approximately a decade earlier using monoclonal antibodies (mAbs) (Maecker *et al.*, 1997 and Wright *et al*, 1994). Tetraspanins have a sequence homology that distinguishes them from other proteins with four transmembrane regions. The sequence homology is comprised of four, six, or eight cystein residues with a CCG motif in the larger EC loop (Maecker *et al.*, 1997 and Wright *et al*, 1994). CD9 is widely expressed in many different tissues, but is down regulated in a variety of human cancers. Within the shorter extracellular region there are putative N-linked glycosylation sites that are believed to be present during differentiation (Cha, 2000). It has been shown that N-linked glycosylation of CD9 and endogenous ganglioside GM3 (NeuAc alpha 2-->3Gal beta 1-->4Glc beta 1-->1Cer) synthesis contribute to the suppression of malignancy and to the promotion of apoptosis in Chinese hamster ovary (CHO) cells (Ono, *et al.*). Similar results were observed with other tetraspanins (Ono, *et al.*). The mechanism behind this biological phenomenon is not well understood but the involvement of these glycoproteins and the surrounding endogenous gangliosides in cell motility and survival is noted.

Tetraspanins can form heterobimolecular complexes with integrin, epidermal growth factor receptor (EGFR) and heparin binding epidermal growth factor-like growth factor (HB-EGF) (Berditchevski *et al.*, 1996). Ectodomain shedding of HB-EGF is an important mechanism to regulate the biological activities of membrane proteins. Umata *et al.,* 2001 looked into the signaling mechanism of the ectodomain shedding of pro-heparin binding epidermal growth factor-like growth factor (pro HB-EGF). Pro HB-EGF is more than the precursor of the soluble form of HB-EGF. It is also biologically active such that it forms complexes with both CD9 integrin and EGFR. HB-EGF has also been shown to be a receptor for diphtheria toxin while associated with CD9 (Cha *et al*., 2000; Iwamoto *et al.,* 2000; Iwamoto *et al.,* 1994). Loss of CD9 association lessens HB-EGF affinity for diphtheria toxin and perturbs EGFR signaling (Cha *et al*., 2000; Iwamoto *et al.,* 2000; Iwamoto *et al.,* 1994), suggesting HB-EGF/EGFR signaling may be dependent upon CD9/HB-EGF interactions.

It has been suggested that CD9 may play a role in LIF-mediated maintenance of undifferentiated mouse ES cells and in cell survival. Mouse embryonic stem cells can proliferate indefinitely in an undifferentiated state in the presence of leukemia inhibitory factor (LIF), or differentiate into all three germ layers upon the removal of this factor (Baharvand *et al.,* 2004; Saito *et al.,* 2004). To determine cellular factors associated with self-renewal of undifferentiated ES cells, Oka *et al* screened genes that are expressed in undifferentiated ES cells and down-regulated after incubating these cells in a differentiation medium without LIF for 48 hours. Tetraspanin CD9 mRNA expression was high in undifferentiated ES cells and decreased shortly after cell differentiation (Oka *et al.*, 2002). Immunohistochemistry analysis confirmed that plasma membrane-associated CD9 was expressed in undifferentiated ES cells but low in the

differentiated cells (Oka *et al.*, 2002). The addition of LIF to differentiating mouse ES cells re-induced mRNA expression of CD9, and this was accompanied with a re-appearance of undifferentiated ES cells (Oka *et al.*, 2002). Furthermore, the LIF/STAT3 pathway is critical for maintaining CD9 expression and activation of STAT3 induced the expression of CD9 (Oka *et al.*, 2002). Addition of anti-CD9 antibody blocked ES cell colony formation and reduced cell viability (Oka *et al.*, 2002). The decrease in cell viability after treatment with the anti-CD9 antibody was confirmed by positive propidium (P.I.) staining of cells, suggesting cell death.

It has been shown that human anti-CD9 monoclonal antibody ALB6 can inhibit cell proliferation, reduce cell viability and induce morphological changes specific to apoptosis and also molecular changes, as evidenced by TUNEL and annexin-V staining (Murayama *et al.*, 2004). ALB6 anti-CD9 antibody activated the c-Jun NH2-terminal kinase/stress-activated protein kinase (JNK/SAPK) and p38 mitogen-activated-protein kinase (MAPK) within 5-15 minutes, as well as caspase-3 within 24-48 hours (Murayama *et al.*, 2004). It is worth mentioning that ALB6 induced tyrosine phosphorylation of the p46 Shc isoform specifically and that the overexpression of its dominant-negative form completely suppressed the ALB6-induced activation of JNK/SAPK, p38 MAPK and caspase-3, resulting in the inhibition of apoptotic cell death (Murayama *et al.*, 2004).

In this study, we hypothesis and demonstrate that soluble HBEGF can rescue mouse embryonic stem cell lines from anti-CD9 antibody induced apoptosis by suppressing MAPK signaling.

MATERIALS AND METHODS

Reagents and antibodies

An anti-CD9 KMC8 and isotype control was purchased from Pharmingen International (San Diego, CA). The antibodies against ERK 1/2, phosphorylated ERK 1/2 , SAPK/JNK, phsphorylated SAPK/JNK, p38 MAPK, phosphorylated p38 MAPK, SRC, phosphorylated SRC, PLCγ,, c-CBL, GRB, cleaved caspase-3, cytochrome c, phosphorylated EGFR (Tyr 845, 1068 and 1173) and PY-100 were purchased from Cell Signaling Technology, Inc. (Beverly, MA). EGFR (Tyr 1086) antibody was purchased from Sima-Aldrich (St. Louis, Mo). EGFR (Tyr 1148) antibody, p38 MAPK inhibitor PD 169316 and recombinant HB-EGF was obtained from Calbiochem (Darmstadt, Germany). Mouse anti-CD37 was purchased from US Biological (Swampscott, MA). Recombinate Mouse EGF was purchased from Cell Sciences (Canton, MA). DAPI was ordered from KPL (Gaithersburg, MD).

ES Cell Culture

As described in Hamazaki (2001), mouse ES cell lines R1 (a gift from S. Dalton, Athens, Georgia, USA) and D3 (a gift from S. Dalton, Athens, Georgia, USA) were maintained on gelatin-coated dish in media containing 1000 U/ml recombinate mouse LIF (ESGRO; Chemicon International, Temecula, CA), 20% fetal calf serum, Knockout serum replacer (Gibco, BRL, Grand Island, NY), 2mM L-glutamine, 100 U/ml penicillin/streptomycin, (VWR International, Bridgeport, New Jersey), 2mM sodium pyruvate (VWR International, Bridgeport, New Jersey), 0.2 mM 2-mercaptoethanol (Invitrogen, Carlsbad, CA), and DMEM (VWR International,

Bridgeport, New Jersey). Briefly, mouse ES cells were washed three times with phosphate-buffered saline (PBS) and resuspended in ES cell media. Cells were cultured on Petri dish at a density of 2 x 10^4 cells/cm^2.

Immunoprecipitation and Westernblot Analysis

Cells were lysed using lysis buffer containing 0.5μM 3-[(3-cholamidopropyl)-dimethylammonio]-1- propanesulfonate (CHAPS (Calbiochem, San Diego, CA) detergent, 100 mM Tris (Sigma-Aldrich, St. Louis, Mo), phosphatase inhibitor cocktail set II (Calbiochem, San Diego, CA), protease inhibitor cocktail tablets-complete (Roche, Penzberg, Germany). Immunoprecipitations with the indicated antibodies were incubated with 50μg of cell lysate for 1hr followed by the addition of protein-G-Sepharose for an additional hr. at 4^{o}C. The system used for western blot analysis was the mini-Proten II (Bio-Rad, Hercules, CA). Proteins were separated on pre-cast Tris-HCL gels (4-15% gradient (Bio-Rad, Hercules, CA)) at 30 milli-amps (2 gels) for 1 hour. Proteins were transferred to nitrocellulose membranes (0.4 μm (Bio-Rad, Hercules, CA)) at 250 milli-amps for 1 hour. Membranes were then blocked in 5% bovine serum albumin (BSA (Pierce, Rockford, IL)) in PBS. The appropriate primary antibody was incubated with the membrane in 1% BSA (Pierce, Rockford, IL) in PBS at room temperature for 1 hr. Membranes were washed for 30 minutes in wash buffer (Bio-Rad, Hercules, CA). HRP-labelled secondary antibody was added to membrane incubated in 1% BSA (Pierce, Rockford, IL) in PBS at room temperature for 1 hr. After a 30 minute washing membranes were incubated in ECL substrate for 1 minute (Pierce, Rockford, IL). Filters were placed between plastic sheets and exposed to Ektar Kodak film for 5 seconds to 2 minutes prior to development of signal in an x-

ray film developing machine (AGFA, Gevaert, NV). Developed x-ray films were stored away from exposure to light.

Immunofluorescnce Microscopy

Mouse ES cells (R1 and D3) were cultured on gelatin-coated chamber slides. After washing with PBS, cells were fixed with 2% paraformaldehyde (PFA) at room temperature for 15 minutes. Cells were washed 2x with PBS and treated with 0.5% Triton X/PBS for 5 minutes, washed, then blocked with 2% PBS for 1hr at room temperature. Cell were incubated with primary antibody overnight. After two washings, cells were incubated with the appropriate fluorescein isothiocyanate antibody (Jackson Immunoresearch Laboratories, West Grove, PA). After two washings with PBS, cells were mounted by vectasheild (Vector Labs, Burlingame, CA).

Transfection of mouse ES cells

R1 and D3 mouse embryonic stem cells were cultured on gelatin-coated dish in media containing 1000 U/ml recombinate mouse LIF (ESGRO; Chemicon International, Temecula, CA), 20% fetal calf serum, Knockout serum replacer (Gibco, BRL, Grand Island, NY), 2mM L-glutamine, 100 U/ml penicillin/streptomycin, (VWR International, Bridgeport, New Jersey), 2mM sodium pyruvate (VWR International, Bridgeport, New Jersey), 0.2 mM 2-mercaptoethanol (Invitrogen, Carlsbad, CA), and DMEM (VWR International, Bridgeport, New Jersey) in a humidified atmosphere of 10% CO_2 in air at 37^{o}C. To establish a stable CD9 knock down R1 ES cell line, cells were transfected with 3 vector-based siRNA constructs to find the most potent one, using lipofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer's

directions. The transfected cells were selected based upon their ability to grow in the presence of 20μg/ml hygromycin B.

Results

Antibody ligation of CD9 induces apoptosis in mouse embryonic stem cells.

As mentioned previously (Masahiro *et al.*, 2002), CD9 antibody interferes with the formation and viability of ES cells at a concentration of 2 x 10^3 cell/ml/ In an effort to determine if cell death is a result of apoptosis, we looked for the presence of cytosolic cytochrome c in the and cleavage of caspase-3. A major route to caspase-3 activation and apoptosis results from cellular stresses, which include, heat stress, osmotic stress, cytokine deprevation or DNA damage that cause permeabilization of the outer membrane of the mitochondria resulting in the release of cytochrome c into the cytosol. In the cytosol, cytochrome c promotes the assembly of caspase-activating complexes referred to as apoptosomes which promotes activation of downstream effector caspases and proteases. Caspases may feed back directly on the mitochondria to amplify cytochrome c release loss of mitochondrial stability promoting apoptosis. We treated R1 and D3 mouse ES cells the anti-mouse-CD9 mAb KMC8. As shown in Fig 3A, B, the ligation of CD9 induces the release of cytochrome c and the activation of caspase-3 indicating cell death to be apoptosis. Furthermore in both R1 and D3 apoptosis, as indicated by the presence of cytosolic cytochrome and activated caspase-3, was specific to the KMC8 antibody and was not induced by the anti-CD37 antibody or control antibody.

Furthermore, because it has been reported that anti-CD9 antibodies impede juxtacrine growth factor activities of HB-EGF, which may be involved with the promotion of rescue and

juxtacrine organizations (RAJOs) and promote cell survival and proliferation, we have investigated whether HB-EGF and epidermal growth factor (EGF), ligands of epidermal growth factor receptor (EGFR), is able to rescue cells from KMC8-antibody induced apoptosis. HB-EGF reduced cytochrome c release and activation of caspase-3 (Fig. 3A, B) by 2.5 fold (Fig 3C). Although reduced, EGF was not as effective in stifling apoptosis (Fig. 3A, B), (Fig 3C). HB-EGF alone does not induce apoptosis (Fig. 3A, B), (Fig 3C).

HB-EGFR and p38 MAPK inhibitor perturbs anti-CD9 antibody stimulated phosphorylation of tyrosine residues 1147 and 1173 on EGFR.

Our investigation has revealed CD9 antibody ligation induces EGFR phosphorylation of tyrosine 1148 and 1173 (Fig. 3D). Our data indicates the addition of HB-EGF and PD 169316 (p38 MAPK inhibitor) to cells treated with anti-CD9 antibody suppresses the activation or phosphorylation of EGFR tyrosine residue 1173, perturbing apoptosis. EGFR tyrosine residue 1148 remained phosphorylated, suggesting tyrosine 1173 phosphorylation is necessary for anti-CD9 antibody induced apoptosis (Fig. 3E). Anti-CD9 antibody did not induce the phosphorylation of other EGFR tyrosine residues known for their role in signal transduction. Specifically, the phosphorylation of EGFR tyrosine residue 1148 and 1173 leads to the activation of the MAPK/ERK signaling cascade (Hackel, 1999).

Anti-CD9 antibody induced EGFR phosphorylation leads to the phosphorylation of p46 Shc isoform in mouse embryonic stem cells.

CD9 antibody appears to activate Shc p46 within 5 minutes but phosphorylated Shc p46 returns to original levels within 24 hrs (Fig. 3F). This activation is a result of phosphorylation of

specific tyrosine residues on EGFR (Fig. 3D, E). As a result of anti-CD9 antibody ligation, tyrosine 1148 and 1173 on EGFR become phosphorylated and recruits Shc for interactions with EGFR. Within 5 minutes, p46 Shc isoform becomes phosphorylated when treated with anti-CD9 antibody alone and in the presence of the KMC8 mAb + HB-EGF and P38 inhibitor. Phosphorylation of p46 Shc isoform returns to untreated levels within a 24hr time frame. Control antibody did not produce phosphorylated p46 Shc isoform.

HB-EGF and p38 inhibitor prevents apoptosis through the suppression of p38 MAPK phosphorylation after antibody ligation of CD9.

MAPK signaling pathways have been implicated in pro-apoptotic and pro-survival cell signaling. It has been reported that, in cancer cell lines, MAPK activity is involved in apoptosis induced after antibody ligation of CD9. We further investigated the question of whether anti-CD9 antibody-induced apoptosis is correlated with the activation of MAPK pathways in mouse embryonic stem cells. Furthermore, if MAPK activity is involved in anti-CD9 antibody induced apoptosis in mouse ES cells, does HB-EGF and p38 inhibitor cell rescue occur as a result of suppression of MAPK signaling. As shown in Fig. 3G, within 5 min, the activation of JNK p46 and p52 and ERK p42 were markedly less with the p38 inhibitor treatments compared to the other treatments. Anti-CD9 antibody activation of p38 MAPK was completely suppressed by HB-EGF and p38 MAPK inhibitor (Fig. 3H). Therefore, these results suggest that anti-CD9 antibody activates MAPK signaling pathways. Over the duration of an hour, HB-EGF and p38 MAPK inhibitor suppresses activation of p38 MAPK signaling preventing apoptosis. Furthermore, p38 MAPK signaling appears to be necessary for anti-CD9 induced cell death. Cell cycle protein CDK2 was used as loading control.

Tyrosine phosphorylation of signaling-transducing proteins induced by CD9 ligation

It has been shown that antibody ligation of CD9 induces tyrosine phosphorylation in platelets (REF). We investigated the phosphorylation of EGFR signal transduction proteins in mouse ES cells after KMC8 antibody treatment. Treatment did not induced tyrosine phosphorylation of PLCγ, Cbl or Src (not shown), but the contrary was observed with GRB, a result of interaction with EGFR tyrosine residue 1173 and Shc (Fig. 3G) (Ref).

CD9 is essential for the survival of R1 and D3 mouse embryonic stem cells

To determine the necessity of CD9 for cell survival, we transcfected 3 vector-based siRNA constructs into R1 and D3 mouse embryonic cells. As shown in Fig. 3I, although transfection was successful as indicated in panel A, as indicated in panel C, a stable CD9 knock down was not achieved. In the suppression of CD9 expression, cells loss their ability to maintain their membrane integrity over a fifteen day period. Cells that did survive the knock down of CD9 experienced morphological changes (Fig. 3I panel D) and were manually selected and passaged and were found to be unviable. These images are a representation of three repeats (Fig. 3I). In summary, CD9 appears to be essential for R1 and D3 mouse ES cell survival.

Discussion

The primary purpose of this research was to determine the role of CD9 in the suppression of apoptosis. It has previously been shown antibody ligation of CD9 induces apoptosis in mouse and human embryonic stem cells (Masahiro, 2002). Additionally, it has also been shown to induce apoptosis in several human cancer cell lines and perturb cell migration (Murayama,

2004). In an effort to further understand their functions anti-tetraspanin antibodies have been implemented with numerous cell types, including embryonic stem cells. It is noted, antibodies against tetraspanin CD9 induce cell death, specifically apoptosis (Murayama, 2004; Oka, 2002), although the signaling cascade in embryonic stem cells is not well defined. Our data supports these previous findings, however, at higher cell concentration (2 x 10^4) apoptosis was not observed (Data not shown). At lower cell concentration (2 x 10^3), cell death was perturbed by the addition of HB-EGF and p38 MAPK inhibitor (PD169316). Although similar results were observed with other growth factors, this recovery effect was specific to HB-EGF and PD169316, suggesting HB-EGF and PD169316 suppress apoptosis signaling and/or stimulates signaling cascades that promote cell survival. Regarding HB-EGF, suppression of apoptotic signaling may occur at its target receptor EGFR, upstream of the MAPK signaling pathway. PD169316 has not been shown to directly effect the EGF receptor, rather is prevents the cleavage of Bid (Ortiz *et al.*, 2001) a BH3-only protein that is instrumental in the release of cytochrome c from the mitochondria, as a result of environmental stresses such as but not limited to osmotic, heat or oxidative stresses.

Our investigation has revealed CD9 antibody ligation results in EGFR phosphorylation on tyrosine 1148 and 1173. This may result from direct associations between CD9 and EGFR or through indirect interaction between CD9, Integrins, HB-EGF or EGFR, which have all been associated with MAPK signaling (Ref). It is noteworthy to mention we have found these proteins form a multimeric complex, as they do in other cell types (Ref). Our data indicates the addition of HB-EGF to cells treated with anti-CD9 antibody suppresses the activation or phosphorylation of EGFR tyrosine residue 1173, perturbing apoptosis. EGFR tyrosine residue 1148 remained phosphorylated, suggesting tyrosine 1173 phosphorylation is necessary for anti-

CD9 antibody induced apoptosis (Fig 5b). Anti-CD9 antibody did not induce the phosphorylation of other EGFR tyrosine residues known for their role in signal transduction. Specifically, the phosphorylation of EGFR tyrosine residue 1148 and 1173 leads to the activation of the MAPK/ERK signaling cascade (Hackel, 1999). In response to extracellular signal, the SH2 and PTB domains of Shc interact with activated receptors on EGFR and Src, leading to the phosphorylation of Shc on Y-239, 240 and 317. Sch exist in p46, p52 and p66 isoforms, which are produced through differential splicing (Rozakis-Adcock, 1999). Our data shows antibody ligation of CD9 results in the phosphorylation of Shc, which occurs exclusively on the p46 isoform. This phenomenon occurs within the initial five minutes of antibody treatment, but subsides inside of 24hrs. Cells with the phosphorylated Shc isoform eventually underwent apoptosis. As we previously suggested, HB-EGF provides an apoptotic rescuing effect that is growth factor specific. HB-EGF did not prevent the apoptotic inducing phosphorylation of the Shc isoform p46, although cell survival was evident, suggesting Shc p46 phosphorylation may be necessary but not sufficient to induce cell death. Phosphorylation of EGFR at Y-1148 and Y-1173 is necessary for recruitment of Shc to the receptor for subsequent phosphorylation and activation. HB-EGF and PD 169316 suppresses the activation of Y-1173 only, therefore Y-1148 remains in an active state to phosphorylate Shc p46.

The MAPK (Erk 1/2) signaling pathway plays a critical role in the regulation of cell growth and differentiation (Crews, 1992). Growth factors, such as, HB-EGF, stimulates the activation of Raf/MEK/ERK 1/2 protein kinase cascade. Additionally, receptor tyrosine kinases (RTKs), integrins and ion channels are also involved with activating the MAPK/Erk signaling cascade. Phosphorylated Erk has several downstream targets, including c-Myc, which promotes cell proliferation in the presence of cdk inhibitors and induces apoptosis when overexpressed in

serum deprived cells (Alevizopoulos , 1997; Vlach, 1997). Our data shows activation of Erk 2 (p42) as a result of anti-CD9 antibody treatment. A greater amount of phorsphorylation was observed in cells treated with anti-CD9 antibody and HB-EGF, while p38 inhibitor promoted Erk phosphorylation. We conclude increased Erk p42 phosphorylation is required for cell rescue of anti-CD9 antibody induced apoptosis.

Phospho-p38 MAPK is also involved in the progression of apoptosis. Here, we show treatment with anti-CD9 antibody induces phosphorylation of p38, whereas, treatments with anti-CD9+HB-EGF or p38 inhibitor prevents this phenomena. p38 inhibitor prevents phosphorylation by blocking the ATP site on p38. We believe HB-EGF and p38 inhibitor work similarly in the suppression of p38 phosphorylation although the mechanisms by which HB-EGF suppresses phosphorylation is not yet determined.

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CHAPTER 4

DISCUSSION AND FUTURE DIRECTION

Our investigation has shown that CD9 is involved in the suppression of apoptosis. CD9 has been previously shown to associate with other molecules such as HB-EGF, integrin and EGFR (Berditchevski *et al.*, 1996). Furthermore, CD9 association with HB-EGF has been shown to attribute to the HB-EGF sensitivity to diphtheria toxin and EGFR signaling (Cha *et al*., 2000; Iwamoto *et al.,* 2000; Iwamoto *et al.,* 1994). Our study has shown that antibody ligation of CD9 activates EGFR by phosphorylating specific residues (Y-1148 and Y-1173) which leads to the recruitment of Shc. Although Shc has 3 isoforms, only p46 Shc isoform becomes phosphorylated in respons to treatment with KMC8 anti-CD9 antibody. Subsequently, cytochrome c release, caspase-3 cleavage and activation of the p38 MAPK, SAPK/JNK, and ERK pathways occur, leading to apoptosis. However, treatment with HB-EGF and p38 MAPK inhibitor perturbs the release of cytochrome c, caspase-3 cleavage and silences p38 MAPK phosphorylation. The aforementioned correlates with cell survival, suggesting HB-EGF and p38 MAPK inhibitor are capable of compensating for the loss of CD9 function in suppression of apoptosis the results from antibody ligation.

Future directions would include attempting to perturb CD9 suppression of apoptosis in cancer cell types for therapeutic purposes. This would include comparisons of cells treated with multiple anti-CD9 antibodies, growth factors and inhibitors to determine the most effective treatments to induce apoptosis in cancer cell lines.

Through our study we determined that DBA lectin can be used to discriminate mouse ESCs that are in different developmental states. Surface markers CD9, SSEA-1, FA are currently used as indicators of pluripotency (Kucia *et al.,* 2006; Oka *et al., 2002;* Rathjen *et al.,*

1999). Additionally, transcription factors Gbx2 and Oct4, amongst others, are also used (Rathjen *et al.,* 1999). All of the aforementioned down regulate slowly and require limiting techniques. We have shown DBA to be can be a more useful tool to monitor self-renewing mESCs and early differentiation events than markers currently used.

Future direction would include the determination of lectins that recognize ligands on human ESCs, in addition to the several that have been determined (Venable *et al.,* 2005). Furthermore, lectins that define the state of early development in human ES cells have yet to be determined and would have great benefit to isolate cells that are at specific states of development.

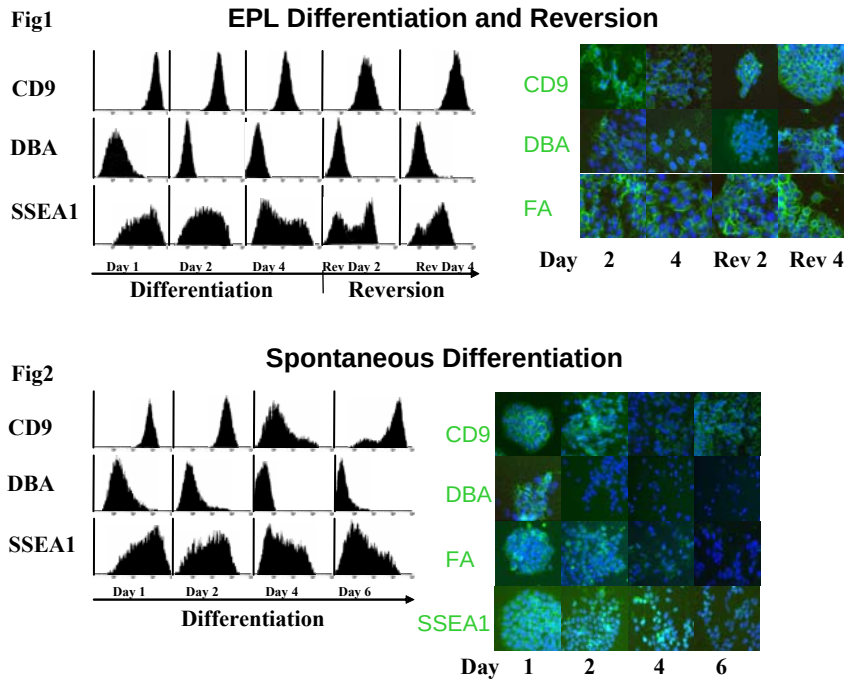


Fig. 1-2 Pluripotent surface markers on ES cells, EPL cells and spontaneously differentiated cells. (A) Mouse spontaneously differentiated ES cells and (B) EPL cells were trypsinized and harvested for flow cytometry and fixed with 4% PFA and permeabilized with 0.5% Triton X-100 for immunocyto staining. For flow cytometry analysis cells were stained with anti-CD9 antibody, DBA lectin and anti-SSEA-1 antibody. Cells were stained with anti-CD9 antibody, DBA lectin, anti-Forssman antigen antibody and anti-SSEA-1 antibody for ESC and spontaneously differentiated cell immunocytochemistry. With respect to the ESC and EPL immuno staining, anti-SSEA-1 antibody was not used.

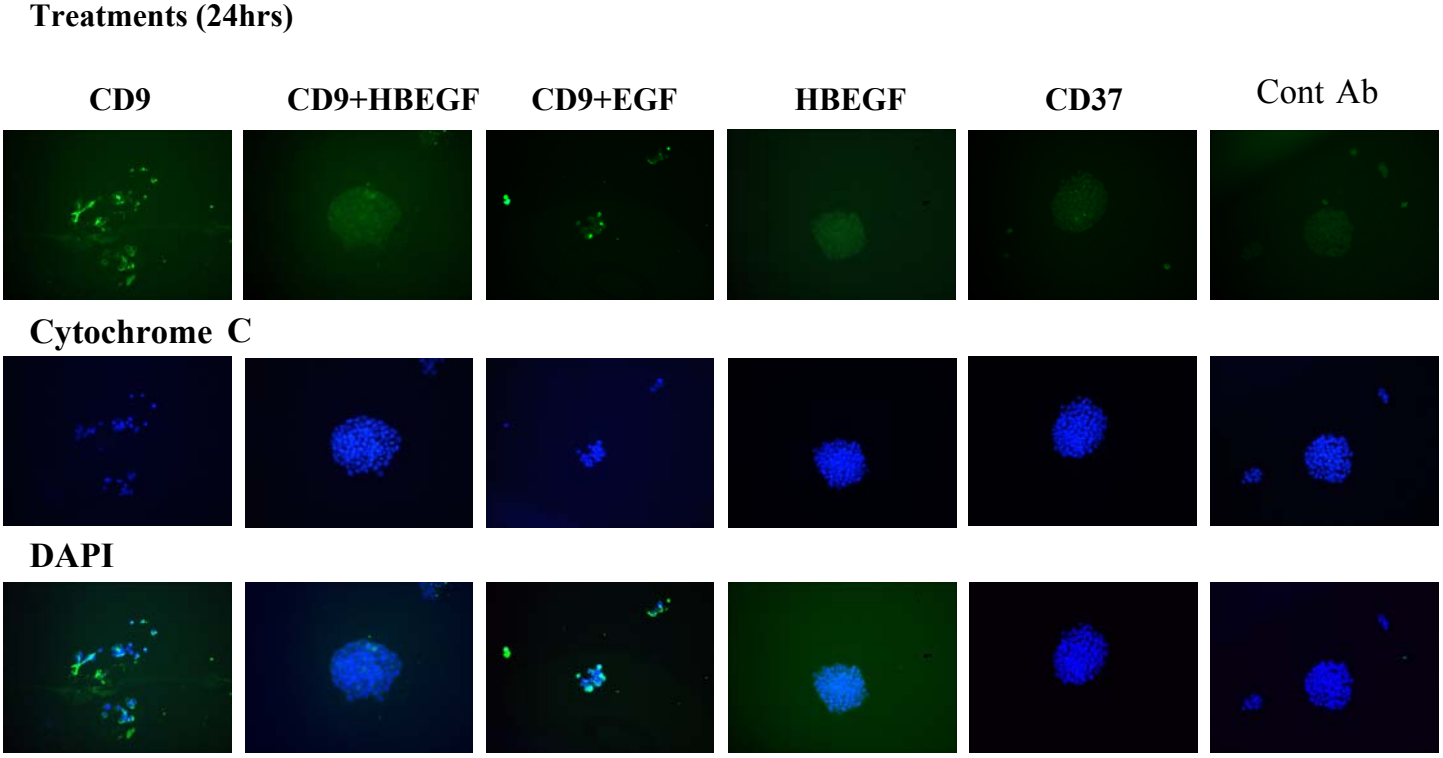


Fig 3A

Fig. 3A. Anti-CD9 antibody treatment causes cytochrome c release.
Cells were trypsinized and plated on 24-well plates coated with gelatin over night at 37^{o}C in 10% CO^{2}. Cells were treated as indicated with CD9 antibody, CD9 antibody+HB-EGF, HB-EGF, CD37 antibody and IgG2a rat control antibody overnight (~24 hour). Cells were washed in PBS 2 x and permeablized with triton x-100 (0.05%) for 5 minutes after 15 min PFA fixation (2-4%). Blocking was performed using 1% BSA in PBS for 1 hour at 37^{o}C. Primary antibody against cytochrome c was add overnight. The appropriate labelled-secondary antibody was added for 30 minutes. Finally, DAPI was added at 1:500 for 3 minutes. Cells were viewed through a flourescent microscope.

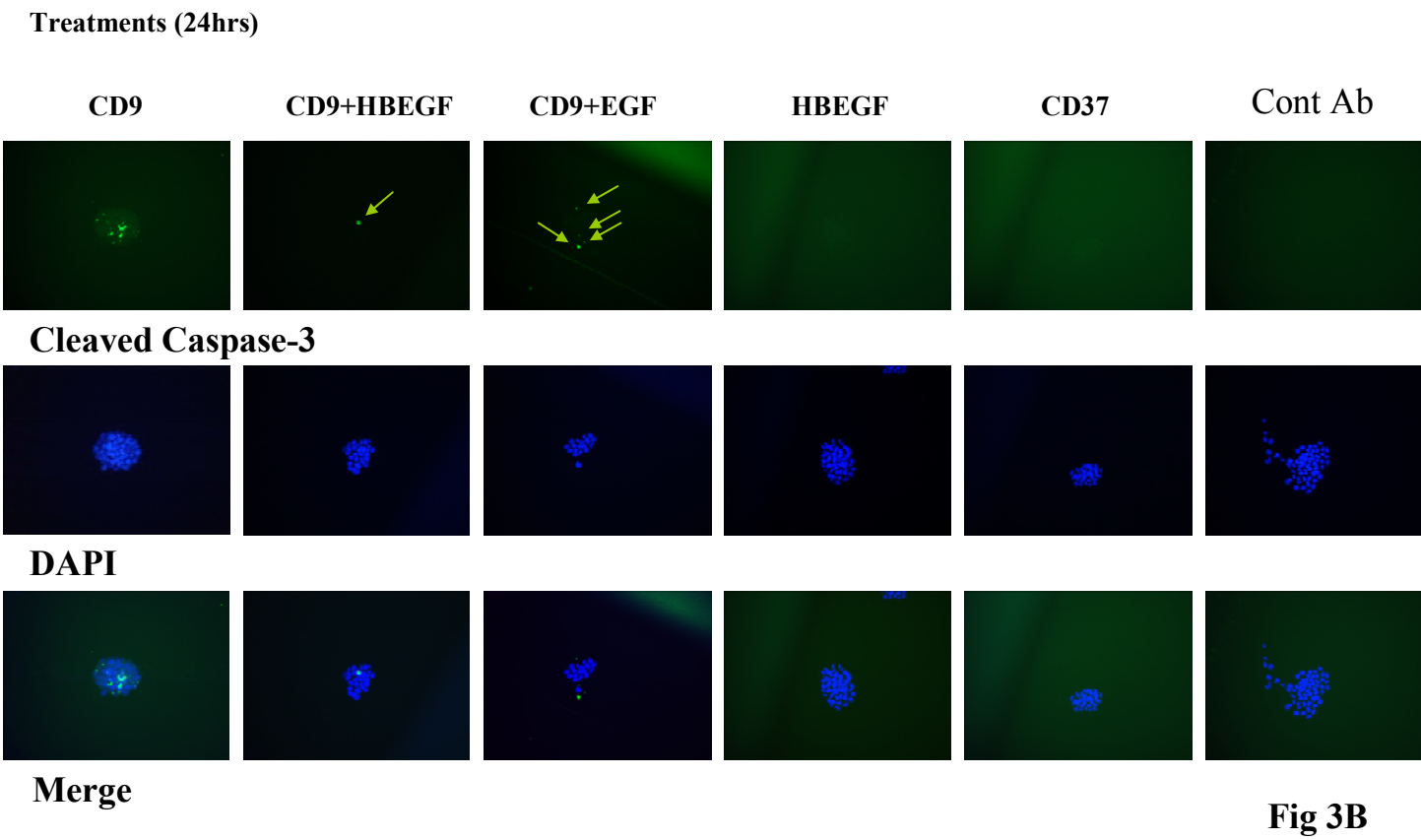


Fig. 3B. Anti-CD9 antibody treatment causes cleavage of caspase-3.
Cells were Trypsinized and plated on 24-well plates coated with gelatin over night at 37^{o}C in 10% CO^{2}. Cells were treated as indicated with CD9 antibody, CD9 antibody+HB-EGF, HB-EGF, CD37 antibody and IgG2a rat control antibody overnight (~24 hour). Cells were washed in PBS 2 x and permeablized with triton x-100 (0.05%) for 5 minutes after 15 min PFA fixation (2-4%). Blocking was performed using 1% BSA in PBS for 1 hour at 37^{o}C. Primary antibody against cleaved caspase-3 was add overnight. The appropriate labelled-secondary antibody was added for 30 minutes. Finally, DAPI was added at 1:500 for 3 minutes. Cells were viewed through a flourescent microscope.

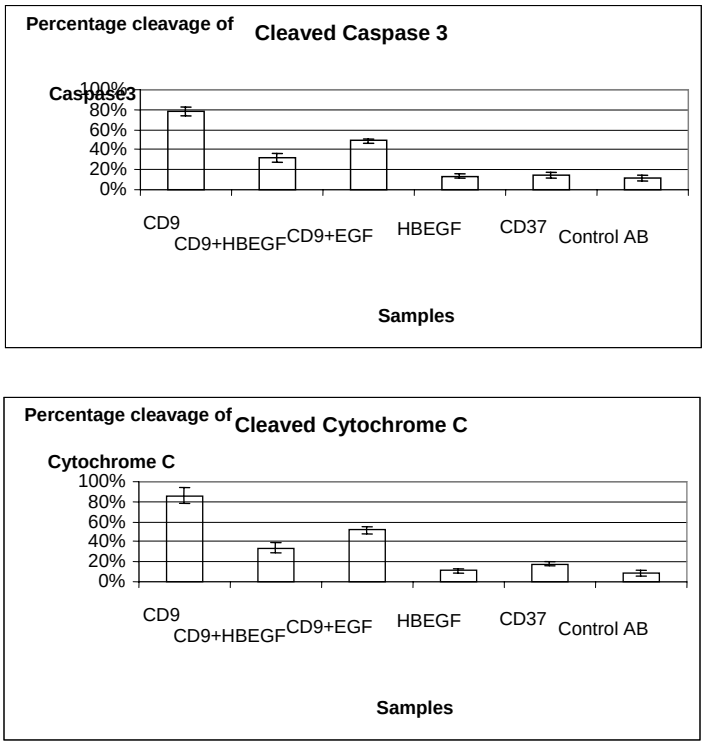


Fig. 3C

Fig. 3C. Group statistics for caspase-3 cleavage and cytochrome c release. Cells were grown at 2 x 10^3 cmxcm on 24 well plates. Cells were treated as indicated with CD9 antibody, CD9 antibody+HB-EGF, HB-EGF, CD37 antibody and IgG2a rat control antibody overnight (~24 hour). Cells were washed in PBS 2 x and permeablized with triton x-100 (0.05%) for 5 minutes after 15 min PFA fixation (2-4%). Blocking was performed using 1% BSA in PBS for 1 hour at 37^{o}C. Primary antibody against cleaved caspase-3 and cytochrome c were add overnight. The appropriate labeled-secondary antibody was added for 30 minutes. Statistical comparison between groups were performed with the Student's *t*-test. Results were derived from triplicate experiments.

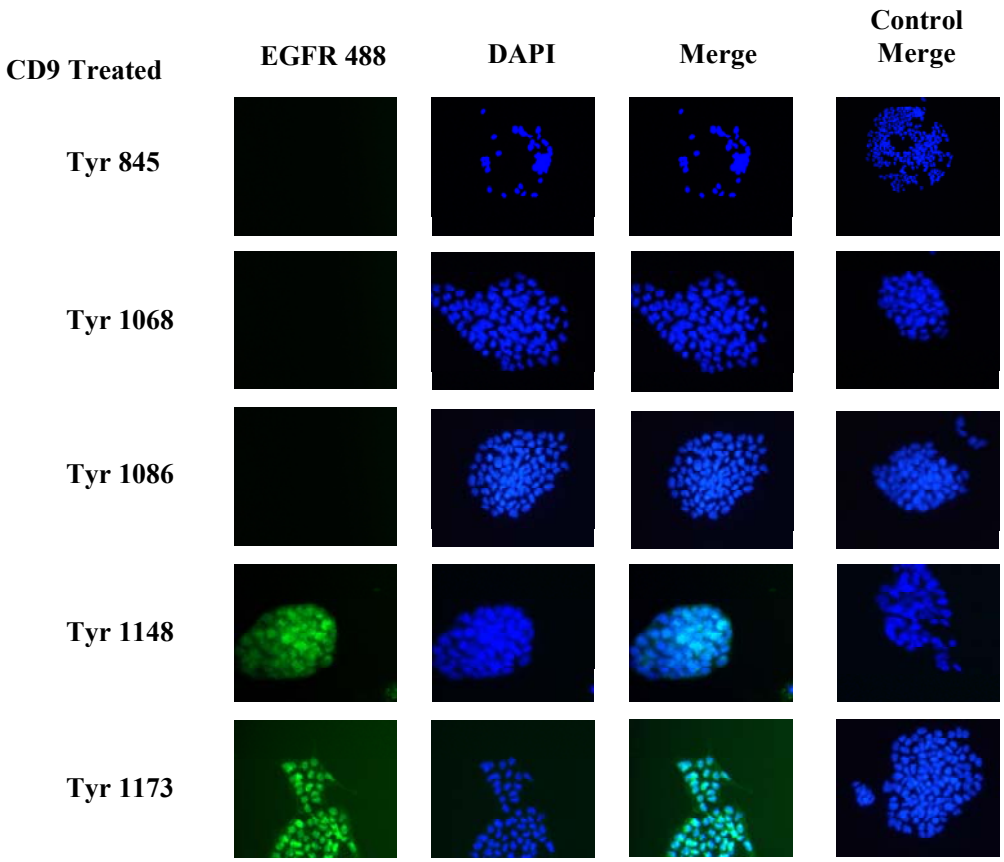


Fig.3D

Fig. 3D. Phosphorylation of EGFR. Cells were Trypsinizedand plated on 24-well plates coated with gelatin over night at 37^{o}C in 10% CO^{2}. Cells were treated as indicated with CD9 antibody, Cells were fixed with PFA, permeablized with triton-x-100 and blocked with 1%BSA in PBS. Anti-EGFR-tyr 845,1068,1086,1148,1173 antibodies overnight. Alexa 488-anti-rabbit secondary was used and DAPI staining was done to identify the nucleus. Cells were viewed under fluorescent microscopic conditions.

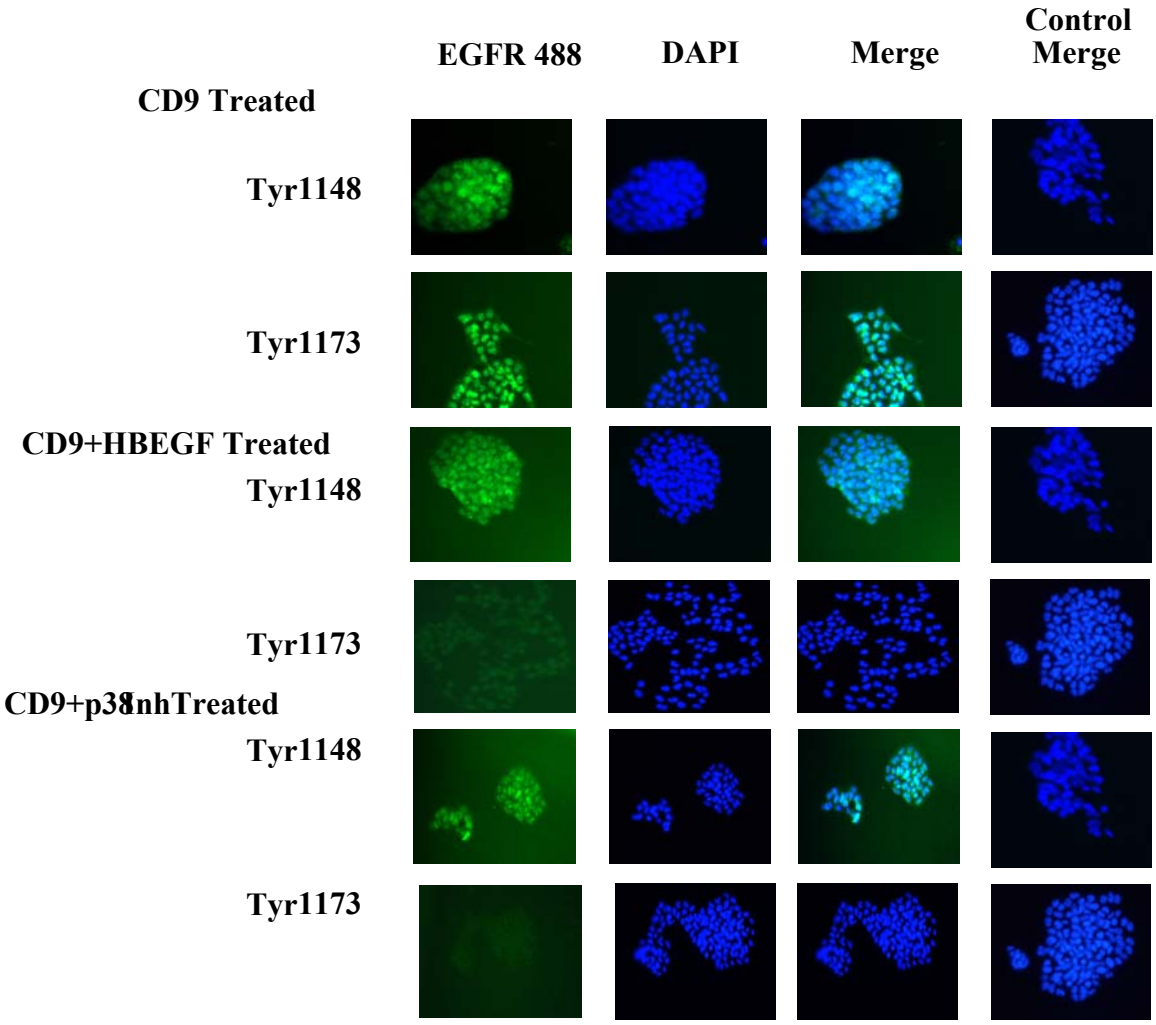


Fig 3E

Fig. 3E. Phosphorylation of EGFR suppressed by HB-EGF and p38 MAPK inhibitor. Cells were Trypsinizedand plated on 24-well plates coated with gelatin over night at 37^{o}C in 10% CO^{2}. Cells were treated as indicated with CD9 antibody+HB-EGF and CD9 antibody+p38 inhibitor for 5 minutes. Cells were fixed with PFA, permeablized with triton-x-100 and blocked with 1%BSA in PBS. Anti-EGFR-tyr 1148,1173 antibodies overnight. Alexa 488-anti-rabbit secondary was used and DAPI staining was done to identify the nucleus. Cells were viewed under fluorescent microscopy conditions.

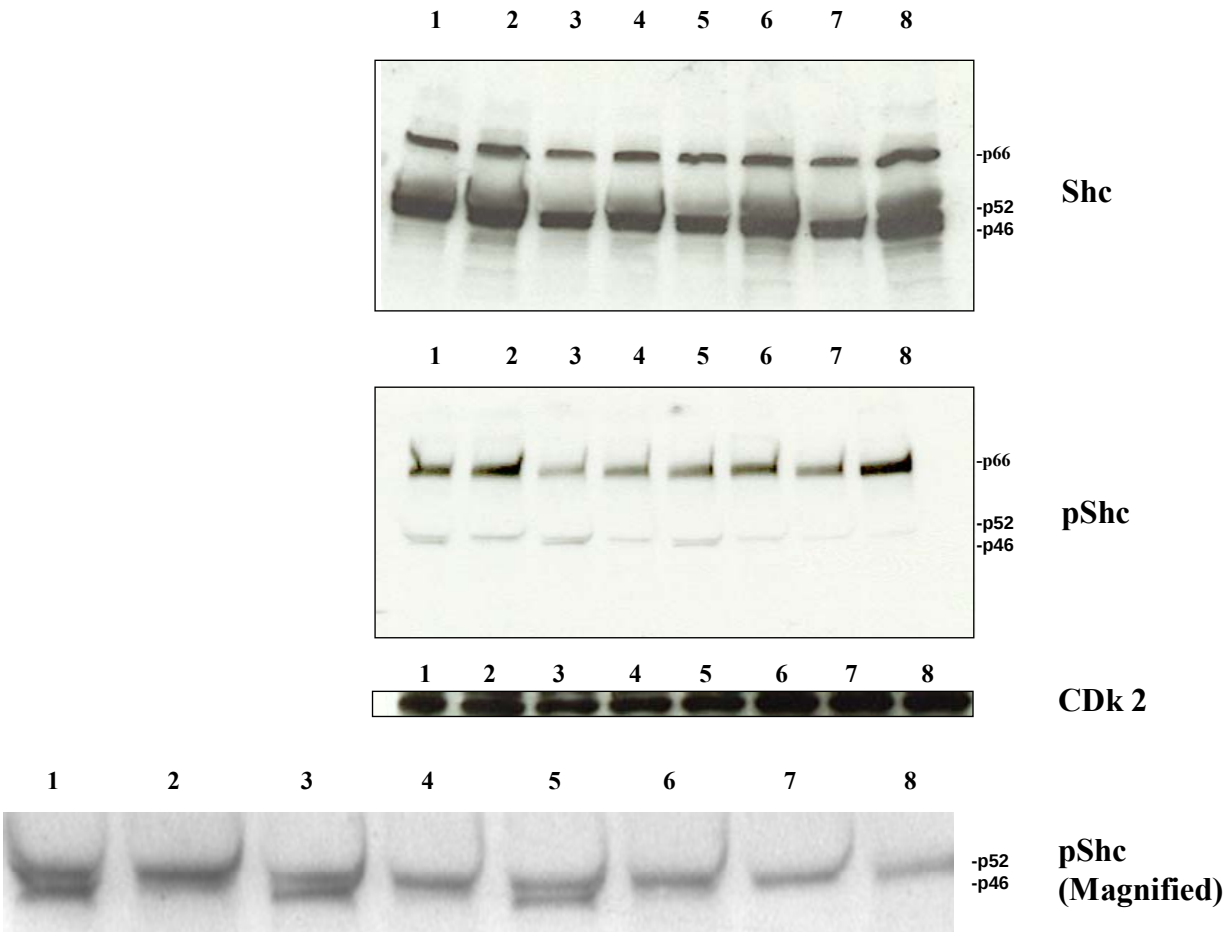


Fig 3F

Fig. 3F. Effects of antibody ligation of CD9 on Shc. ES cells were incubated for 5 min with 50μg/ml KMC8 (1), KMC8+10μg/ml sHB-EGF (3), KMC8+ 10μg/ml p38 inhibitor (5), isotype-matched rat IgG2a, *k* (7) or incubated for 24hrs with 50μg/ml KMC8 (2), KMC8+10μg/ml sHB-EGF (4), KMC8+ 10μg/ml p38 inhibitor (6), isotype-matched rat IgG2a, *k* (8). The adherent cells were harvested and subjected to immunoblots with anti-Shc, anti-phospho-Shc and anti-Cdk2 antibodies.

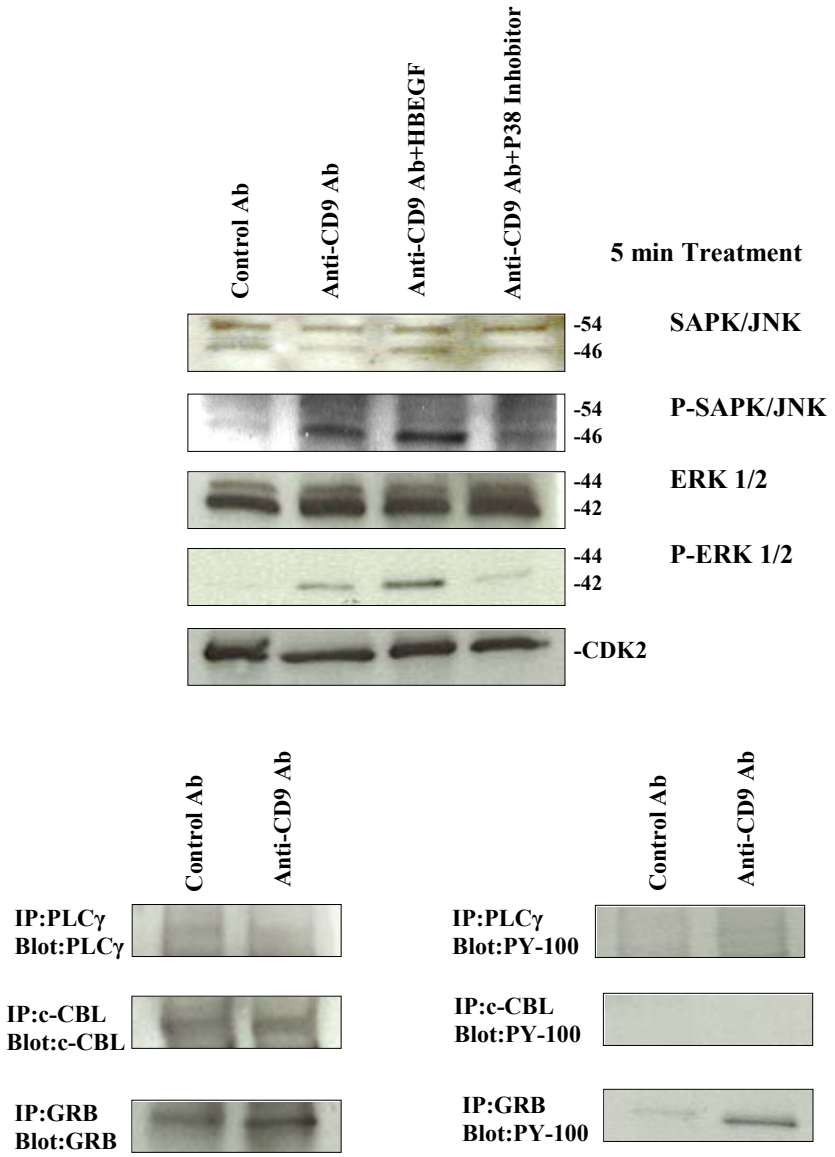
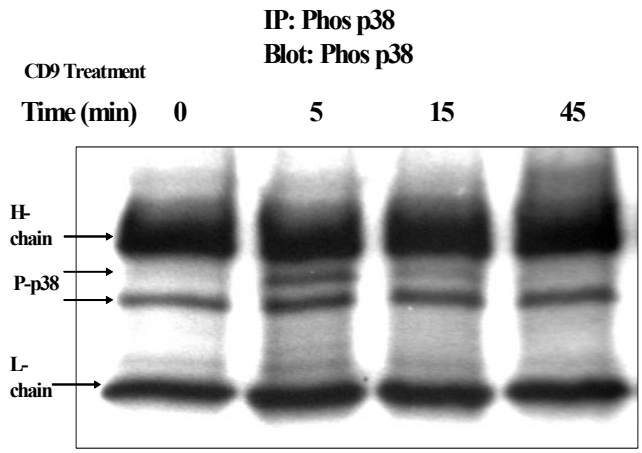
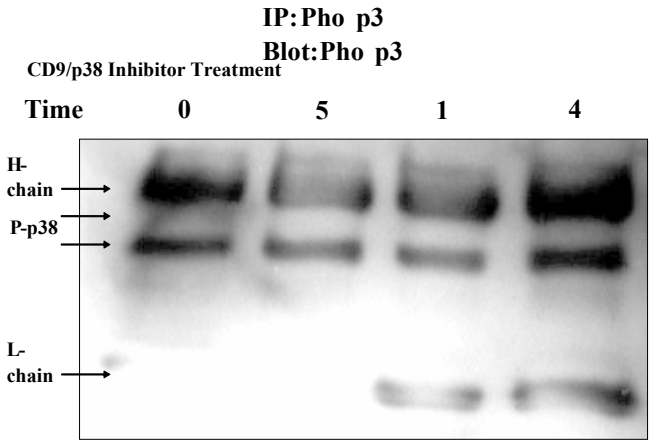
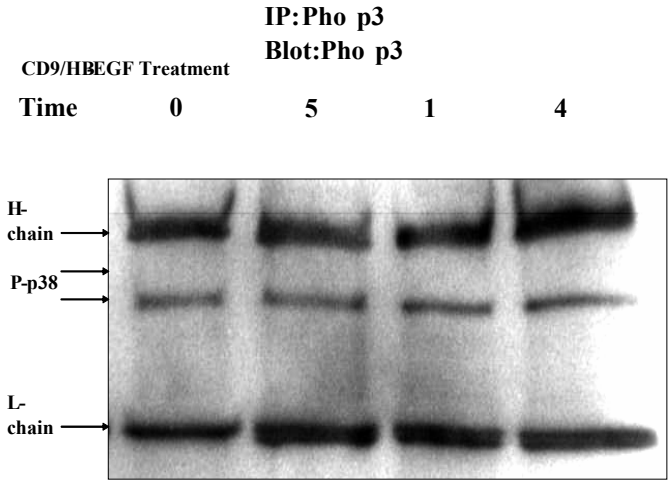


Fig 3G

Fig. 3G. Effects of anti-CD9 antibody on SAPK/JNK and ERK pathway activation. Cells were treated according the labeling for 5 minutes. Cells were lysed with CHAPS buffer, scrapped and quantitated. 50μg of extract was run on 4-15% gradient gel a transferred to nitrocellulose. Anti-SAPK/JNK and anti-phospho-SAPK/JNK antibodies were incubated overnight. HRP-labelled anti-rabbit secondary was used for 1hr. CDK2 antibody was used as loading control. Filters were developed via ECL. Immuno-precipitations were performed using anti-PLCγ, c-CBL and GRB antibodies.







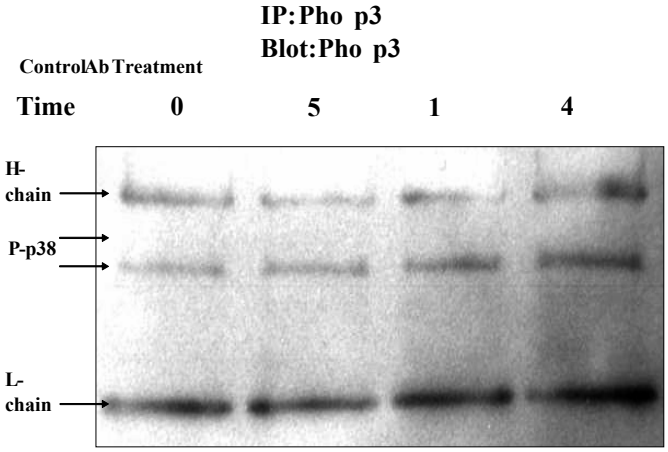
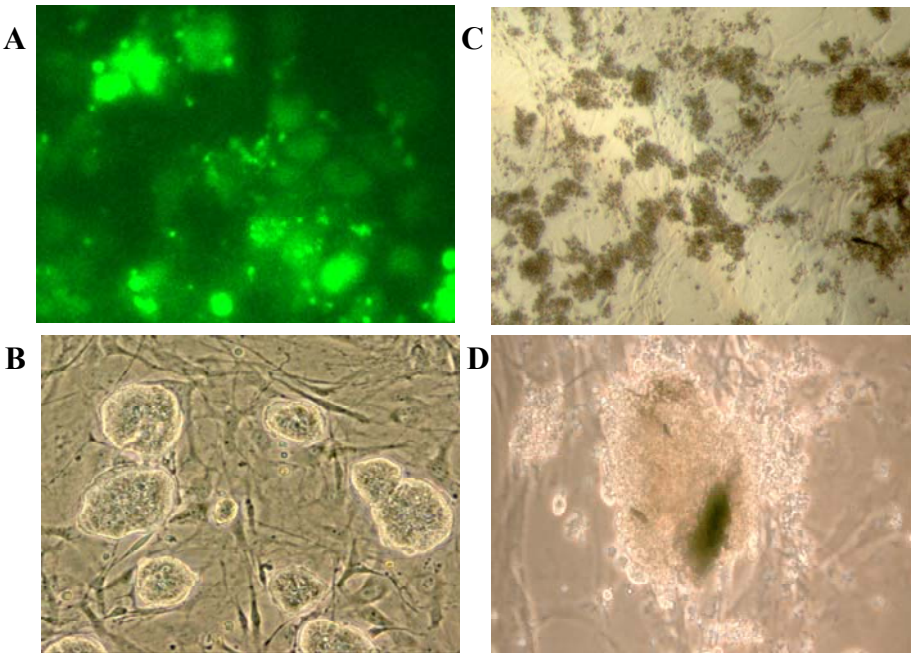


Fig. 3H Effects of anti-CD9 antibody, HB-EGF and p38 MAPK inhibitor on p38 MAPK pathway. Cells were treated with CD9 antibody, CD9 Ab+HB-EGF, CD9Ab+p38 inh and control IgG2a. Cells were lysed with CHAPS buffer, scrapped and quantitated. Immuno-precipitations were performed using anti-pan phospho-p38 antibody. Samples were run on a 4-15% gradient gel and transferred to nitrocellulose membranses. Anti-pan phospho-p38 antibody primary immunoglobulins, overnight. HRP-anti-rabbit Ab was used as secondary. Membranes were developed via ECL.



	siRNA1	siRNA2	siRNA3	Control
Trail 1	0	0	5	45
Trail 2	0	0	3	51
Trail 3	0	0	3	50
Average	0	0	3.6	48.6

Fig. 3I. siRNA transfection Cells were transfected with 3 siRNA constructs and selected with hygromycin for 15 days. Control cells only received fluorescent nucleotides.