Cell-Cell Junction Signaling Regulating DNA Double-Strand Break Repair In Breast Cells

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Cell-Cell Junction Signaling
Regulating DNA Double-Strand Break Repair In Breast Cells

Thesis submitted to the faculty of the Molecular Biology and Genetics Program,
Department of Biochemistry and Molecular Biology, School of Medicine,
Virginia Commonwealth University
in partial fulfillment of the requirements for the
degree of

Master of Science
In
Molecular Biology and Genetics
(Majoring in Biochemistry)

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ABSTRACT

Genomic instability and acquisition of invasiveness through the basement membrane extracellular matrix (ECM) are two major processes for epithelial cell malignancy in breast cancer. DNA double-strand break repair (DSBR) is one of the processes that get misregulated during breast cancer progression. In addition, radiation induced breaks such as those induced during radiation therapy to treat breast cancer patients are repaired by DSBR, rendering this pathway relevant for therapy as well. DSBR can occur either by homologous recombination (HR) or non-homologous end-joining (NHEJ). HR is accepted as the more error-free pathway. HR is regulated by the cell cycle status such that an increase is observed in G2/M, whereas NHEJ is observed throughout the cell cycle.

Previous data show that ECM signaling regulates HR, as well as the kinetics of ionizing radiation (IR) induced complex formation at break sites, or foci kinetics. Both human breast epithelial cell lines and primary mouse mammary epithelial cells were used to show that the ECM receptor β1-integrin is necessary and sufficient in down regulating HR, as well as IR induced foci formation kinetics for the DSBR proteins RAD51, MRE11, and γ-H2AX in single mammary epithelial cells. RAD51 is required for most HR, whereas MRE11 and γ-H2AX function in HR as well as DNA damage signaling. Interestingly, ECM signaling up-regulates HR in cells that have “correct” in vivo-like cell-cell junctions. Based on the observation that single cells and junctioned cells respond to
ECM in exact opposite manner, I hypothesized that ECM signaling may interact with cell-cell junction signaling pathways in regulating DNA repair.

To test this hypothesis, I asked whether the main breast epithelial adherens junction cadherin, E-cadherin, is involved. I blocked E-cadherin function using a monoclonal antibody MB2. The function blocking was demonstrated by the loss of cell-cell junction interactions and observation of increased cell scattering using phase microscopy. I then asked whether blocking E-cadherin altered the expression and localization of proteins related to DNA repair. Indirect immuno-fluorescence showed that in the E-cadherin blocked non-tumorigenic breast epithelial cell line HMT-3522 S1 there is an up-regulation of nuclear γ-H2AX and RAD51, as well as an increase in the proliferation marker Ki67. In non-proliferative MB2 blocked cells there is an upregulation of γ-H2AX and reduced Ki67. Furthermore, in these proliferative and non-proliferative blocked cells we were able to see lower levels of β-catenin near the cell membrane and an increase in its levels inside the cell especially in the nucleus. The latter has been confirmed also by western blot technique comparing the nuclear and cytoplasmic fraction expression. In addition, western blots showed that total RAD51 level was down-regulated by E-cadherin blocking and γ-H2AX levels were found to be higher in proliferative and non-proliferative MB2 treated cells. MB2 treated cells have a higher frequency of HR in the absence of ECM and in the presence of ECM, MB2 blocking abolishes the ECM effect on HR. Furthermore, in the absence of ECM, RAD51 siRNA treated cells down-regulated HR but the absence of RAD51 did not down regulate HR in the presence of...
ECM. I was not able to see any difference in the phosphorylated forms of β-catenin such as Tyr-142, Ser-45 and Tyr-86 that has the ability to enter into the nucleus.

Therefore, E-cadherin was found to block nuclear β-catenin, RAD51 and γ-H2AX in a proliferation-independent manner. E-cadherin also was necessary for ECM to up-regulate HR. The up-regulation of HR by ECM was only slightly dependent on RAD51 suggesting a novel E-cadherin-dependent and RAD51-independent HR component in breast epithelial cells in contact with ECM as they are in vivo in the normal breast tissue.

These experiments will help us to understand the role of E-cadherin and β-catenin in DNA double-stand break repair directly, as well as in combination with ECM signaling. Both alterations in integrin mediated signaling and cell-cell junction integrity contribute to breast cancer progression by rendering breast epithelial cells more invasive. My project will shed light on whether these invasive processes also alter DNA repair and contribute to genome stability. Understanding of the interrelationships among integrin signaling, cell-cell junctions, and genome stability will contribute to understanding normal breast cell processes and open up investigations on how these may go awry in cancer progression.
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INTRODUCTION
INTRODUCTION

Cells create the correct configuration of their microenvironment by secreting and manipulating extracellular matrix (ECM) components. ECM in turn serves as a support for the development of all structures that comprise a functional organism and are also involved in regulating inter-cellular communication. Cells recognize a functional ECM using mostly the major cell receptor, integrins. Integrins play a vital role in the alteration of cellular growth and tumor progression through the regulation of gene expression, apoptosis, cell adhesion, proliferation, migration, angiogenesis[1, 2] and proteinase secretion[3].

EXTRACELLULAR MATRIX (ECM)

In mammary glands, the epithelial cells reside on thin sheet-like structures of ECM known as the basement membrane (BM). Type IV collagen, laminin, heparin sulphate proteoglycans (HSPGs) and nidogen/entactin are some of the major components of BM. Type IV collagen is a major structural protein of BM. They are heterotrimeres composed of three alpha chains. There are 6 genetically distinct alpha chains encoded by genes COL4A1 through COL4A6 located in chromosome 2q36, 13q34 and Xq22 (Figure1) [4].

These alpha chains can arrange into at least three different forms viz., [α1(IV)]α2(IV), [α3(IV)]α4(IV) and [α5(IV)]α6(IV). [α1(IV)]α2(IV) was found to be present ubiquitously in all BMs. Apart from this, α5(IV) and α6(IV) chains were found to be present in the BM of mammary gland [5]. In situ hybridisation and immunohistochemistry have revealed that assembly of α5(IV)/α6(IV) is defective in the invasive stages of breast cancer [6].
Furthermore, many isoforms also exist for these 6 forms of type IV collagen alpha chains further contributing for its diversity.

Each type IV collagen chain consists of three domains: an N-terminal 7S domain, a middle triple-helical domain, and a C-terminal globular noncollagenous (NC1) domain (Figure 2). It is speculated that the triple-helical type IV collagen molecules self associate via their NC1 domains to form dimmers and also at the glycosylated N-terminal 7S domain to form tetramer (lateral association). These interactions form the nucleus of type IV collagen scaffold which will further evolve into a type IV collagen suprastructure representing spider web-like scaffolds with the help of the end to end and lateral associations (Figure 3) [7].

Laminin is a trimer containing α, β, and γ chains and are available in 12 different forms. Laminin is believed to initiate BM scaffold formation at the basolateral surface of the cells. Laminin polymer anchors to the cell surface via site-specific interactions with integrins (especially β1-integrins) and dystroglycans. The type IV collagen scaffold in turn associates with laminin polymers on the cell surface via nidogen/entactin bridging. [7].

Most of the knowledge on signaling and phenotypic consequences of BM was gained from the mouse Engelbrecht Holm-Swarm (EHS) sarcoma tumor secreted ECM used in culture.
These EHS cells produce and accumulate BM components within the tumor tissue that can be easily isolated [7, 8]. We use EHS (BD Matrigel™ Basement Membrane Matrix, Growth Factor Reduced) as ECM for our culture experiments. It is a solubilized basement membrane preparation extracted from the EHS mouse sarcoma, a tumor rich in extracellular matrix proteins that has laminin, collagen IV, heparan sulfate proteoglycans, and entactin/nidogen. BD Matrigel Matrix also contains TGF-β, epidermal growth factor, insulin-like growth factor, fibroblast growth factor, tissue plasminogen activator and other growth factors which occur naturally in the EHS tumor.
**Figure 1: Collagen genes in human.** The location of six different genes that encode for genetically distinct alpha chains of collagen. The collagens are located in pairs and in opposite orientation (head-to-head fashion) on three different chromosomes. The different collagen genes are represented in different colors (Adapted from J Biol chem 268(35), 1993, pp. 26033–6, [4]).
Figure 2. Schematic model of a type IV collagen. Three alpha chains are arranged to form type IV collagen and is represented in the figure as single thick horizontal blue bar for simplicity. It contains three domains. The triple-helical domain consists of ~1400 amino acids (aa) and consists of 22 imperfections within the Gly-X-Y motif. The NC1 domain is ~230 amino acids in length (Adapted from Nat Rev Cancer, 3(6), 2003, pp. 422–33, [7]).
Figure 3. Formation of Type IV collagen network. Two NC1 domain associate to form dimer while four 7S domain associate to form a tetramer. The dimer and tetramer forms the nucleus for the Type IV collagen scaffold formation (Adapted from Nat Rev Cancer, 3(6), 2003, pp. 422–33 [7]).
INTEGRINS

Integrins are the major cell receptors that interact with ECM. They are heterodimeric transmembrane receptors composed of α and β subunits [9]. There are 24 heterodimers of integrin family formed from 18 α and 8 β subunits. Within this integrin family β1-integrins, β2-integrins and αv-containing integrins are the 3 largest groups (Figure 4). Different integrins can bind to different ECM components while some integrins can bind to many ECM components. For instance, α1β1, α2β1, α5β1 integrins bind to collagens, α6β4, α3β1 integrins binds to laminins and α8β1 binds to fibronectin, vitronectin and nephronectin. Furthermore, the interactions between integrins and BM components are altered when there is a structural change in BM components. For example, α1β1 and α2β1 integrins bind to collagen IV and promote proliferation and migration in cells. But when the collagen is degraded by MMP, αvβ3 integrin binds to the denatured collagen IV but not α1β1 and α2β1 integrins.

Integrins can signal bidirectionally [10]. They can get information from extracellular stimuli to induce intracellular changes (outside-in signaling) or from intracellular stimuli to induce extracellular changes (inside-out signaling). The activation of integrins occurs through inside-out signaling [11]. Normally integrins are expressed on the cell surface in an inactive conformation, unable to productively bind to ECM. Through inside-out signaling, talin binds to the cytoplasmic domain of integrin causing structural changes in the extracellular domain of integrin leading to integrin activation [12]. This activation increases the affinity of individual integrin for ECM ligands. Ligand-bound integrins cluster first into unstable structures called nascent adhesions [13]. A subset of nascent
adhesions progresses to dot like focal complexes, which can mature into larger focal adhesions (FAs) and finally into streak-like fibrillar adhesions [14]. Once integrins are activated and clustered they are able to transmit many intracellular changes by outside-in signaling and this occurs by the recruitment of Rho GTPases, Raf, Ras, FAK, and MAPKs such as extracellular-signal-regulated kinases (ERKs) to the ECM ligand-integrin binding site [15].
Figure 4. Representation of the integrin family. In vertebrates, the integrin family contains 24 heterodimers. Different sets of integrin receptors bind to different components of ECM (Adapted from Cell Tissue Res, 339, 2010, pp. 269–80, [16]).
INTEGRINS AND CADHERINS

Various studies report a correlation between cell-ECM and cell-cell adhesions. In colon cancer, inhibition of E-cadherin caused an up-regulation of $\alpha_5$ integrins [17]. In addition, introduction of a dominant negative mutant E-cadherin into keratinocytes resulted in reduction in expression levels and activity of $\alpha_2\beta_1$ and $\alpha_3\beta_1$ integrins [18, 19]. In another study, introduction of E-cadherin to Xenopus-derived fibroblasts caused downregulation of $\alpha_3\beta_1$ integrin and reduced adhesion to fibronectin and laminin [20]. Furthermore, functionally inhibited-anti-cadherin antibodies have been shown to prevent loss of $\alpha_6$ and $\beta_1$- integrin in terminally differentiated keratinocytes [21].

Various experiments also have shown the effect of E-cadherin on the downstream factors of integrin. For instance, ILK involved in integrin signaling pathway was reported to promote $\beta$-catenin translocation to the nucleus while $\beta$-catenin through its downstream factor TCF/LEF regulates the promoter for cyclin D1 [22, 23]. The above evidences suggest that there is some cross talk between integrin signaling and E-cadherin pathway and are summarized in Figure 5. Previous results show that $\beta_1$-integrin mediated ECM signaling downregulates the homologous recombinational repair of double-strand breaks in single cells but has the opposite effect in junctioned epithelial cells [24], suggesting a cross-talk between cell-cell junction and ECM signaling that functions in double-strand break repair. Here we study cell-cell junction signaling involvement in double-strand break repair, focusing on adherens junctions previously shown to interact with ECM signaling pathways.
Figure 5. Relationship between E-cadherin and integrins in different cell lines ([17], [18, 19], [20], [21], [22, 23]).
**E-CADHERIN PATHWAY**

Cadherins are one of the major proteins involved in cell-cell adhesion by forming adherens junctions (AJ) between cells. Cadherins are also involved in regulating cell shape, segregation, migration, proliferation and differentiation [25]. Within the cadherin family of proteins, E-cadherin is found at the junctions of epithelial cells and mediates cell-cell adhesion through homophilic interactions of their extracellular domains. E-cadherin also mediates contact inhibition of cell growth [26]. A reduced E-cadherin expression was observed in at least 41% of human lobular breast carcinoma cases mainly due to hypermethylation of E-cadherin promoter region [27].

The cytoplasmic tail of E-cadherin was found to interact with various proteins such as β-catenin and alpha-catenin that link E-cadherin receptor indirectly to cell signaling and trafficking [28]. Furthermore, it is proposed that the E-cadherin/β-catenin interaction occurs in the ER (endoplasmic reticulum) and is required for cadherin exit from ER. Normally in the absence of Wnt signal, the cytosolic levels of β-catenin are low due to sequential phosphorylation of serine and threonine residues of β-catenin by GSK3β (glycogen synthase kinase-3β) and CK1 (casein kinase 1) within MDC (multiprotein destruction complex) followed by proteasomal degradation [29]. In the presence of Wnt ligand (secreted lipid-modified signaling protein); the receptor is activated causing MDC to destabilize. As a result, the unphosphorylated β-catenin accumulates in the cytosol and is subsequently translocated into the nucleus where it associates with TCF/LEF (T cell factor/lymphoid enhancer factor) transcription factor. This leads to transcription of Wnt target genes [30] (Figure 6).
In addition, CCN6, a Wnt1 induced signaling protein 3 knock down in benign human mammary epithelial cell lines (HME and MCF10A) led to down regulation of E-cadherin [31].
**Figure 6. The Wnt Pathway.** β-catenin is the main protein involved in the pathway and the fate of this protein changes in the presence and absence of Wnt signaling (Adapted from J. Hülsken, MDC, Berlin [32]).
DOUGRN STRAND BREAK REPAIR AND CELL-CELL JUNCTIONS

Genomic instability is one of the main properties of malignant cell that lead to acquisition of various forms of mutations. To maintain a stable genome, DNA double-strand breaks (DSB) caused by ionizing radiation and DNA damaging chemicals should be repaired. DSBs can be repaired by major repair pathways such as homologous recombination (HR) or non homologous end joining method (NHEJ) (Figure 7). HR is known for its error-free repair when compared to NHEJ [33]. Furthermore, HR is predominant during S and G2 phase while NHEJ is upregulated during G1 phase of the cell cycle [34].

In NHEJ, the DNA ends are processed to produce ligatable ends after which the ends are ligated. In case of HR, the DSBs are first sensed by the MRN complex of proteins. The MRN complex is a hetero-trimeric protein consisting of MRE11, RAD50 and NBS1. MRE11 has DNA-binding domains and a domain with exonuclease activity that can also interact with NBS1. NBS1 also has exonuclease activity and contains domains that interact with MRE11 and ATM. RAD50 has walker A and B. Walker A and B are separated by coiled-coil domains with a zinc-hook motif in the center (Figure 8)[35, 36]. When there is a DSB, MRE11 binds to the broken DNA ends through its DNA-binding domain. NBS1 is recruited to this site as MRE11 contains an NBS1 binding domain. RAD50 through its walker A and B tethers the DNA ends. Interaction of RAD50 with MRE11 stimulates endonuclease activity of MRE11 in order to processes the DNA ends to produce 3’overhangs. Recruitment of ATM as inactive dimer at DSB also occurs after the assembly of MRN complex. Both by RAD50 that increases the local
concentration of DNA ends and NBS1, the ATM gets activated into an active monomer which in turn phosphorylates a number of substrates that are involved in HR and NHEJ, including H2AX to $\gamma$-H2AX by phosphorylating H2AX at ser139 (Figure 9)[35]. $\gamma$-H2AX forms foci at and near the DSBs and also acts as a docking site for the DNA repair proteins.

In addition to the MRN complex, also BRCA1 and CtIP are involved in the activation of HR-mediated repair of DNA in the S and G2 phases of the cell cycle. After end resection the 3' overhangs invade the sister chromatids. These single stranded DNA binds to single stranded binding protein, RPA (replication protein A). RAD51 binds to the RPA bound ssDNA with the help of mediators and forms a nucleoprotein filament. This is followed by strand invasion, synthesis of DNA by DNA polymerase and resolution of junctions (Figure 9)[35].

Apart from the above explained HR that involves RAD51, there is also an RAD51-independent HR that has been widely studied in S. cerevisiae. RAD51-independent recombination pathway (RIRP) was predicted to occur by invasion of 3’end of the broken chromatid into an intact non-homologous chromosome leading to non-reciprocal translocations (Figure 10)[37]. Interestingly, in mammalian cells, a research group while studying the effects of HR on cells expressing mutant RAD51, they were able to see some residual level of HR taking place within these cells suggesting that there could be a RAD51 independent recombination pathway in mammalian cells as well [38].
Figure 7. The double strand break repair pathway. Picture showing the different possibilities by which DNA double strand breaks can be repaired and their mechanisms are explained in diagrammatic manner.
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Figure 10. RAD51-independent HR pathway. Figure shows a possible pathway by which DNA double strand break can be repaired in a RAD51 independent manner (adapted from Genetics, 158(3), 2001, pp. 959-72 [37]).
HUMAN BREAST EPITHELIAL CELL LINE MODEL

As a model for breast epithelial cells, S1 and S1 DR9 cells will be used in the experiments. These breast epithelial cells were derived from a reduction-mammoplasty specimen of a patient with fibrocystic breast disease and grown on a defined medium to obtain HMT-3522 S1 cells (referred to as S1 cells from here on) [39]. S1 cells are immortalized but non-tumorigenic when injected into nude mice [40]. Most importantly for this project, S1 cells are dependent on the Epidermal Growth Factor (EGF) for growth. Leaving out EGF from the growth media arrests the cells mostly in the G1 phase of the cell cycle (Figure 11). Double-strand break repair pathway frequency has been shown to depend on which phase of the cell cycle the cells are in. Therefore, by simply not providing EGF to cells, they can be stopped in G1 phase. HR is high in G2/M phase. But since we use a GFP direct repeat sequence in S1 DR9 cells, we are able to observe intrachromatid recombination events in G1 (Figure 12). This helps us to do experiments on HR frequency that are reproducible as all cells will be in one phase of cell cycle.

S1 DR9 cells are normal S1 cells into which two direct repeats of non-functional GFP gene (DR-GFP) have been integrated [41]. The DR-GFP is integrated in the hprt (hypoxanthine phosphoribosyl transferase) locus. The DR-GFP has a 5's-GFP gene that has a rare I-SceI endonuclease recognition site of 18bp length within it. DR-GFP also has a 3'iGFP gene that has 0.8Kb homology for repairing the I-SceI cleaved s-GFP and to restore its expression through homologous recombination, specifically by non-
crossover gene conversion. This can occur between sister chromatids or by intrachromosomal recombination (Figure 12).

But the GFP gene expression will not be restored if the double-strand break (DSB) is repaired by non-homologous end joining method (NHEJ) (Figure 13). Thus the expression of GFP reporter is used as a measure of homologous recombination. In order to induce the break at the I-SceI recognition site, plasmid pBAS that expresses the I-SceI endonuclease from an actin promoter is introduced into S1 DR9 cells. To compare the transfection efficiency, a plasmid expressing GFP from the same promoter is transfected into cells in parallel under the same experimental conditions.
Figure 11. Percentage of non-proliferative S1 cells at different phases of cell cycle. When the normal S1 cells with cell-cell junctions are treated with growth medium without EGF, mostly the cells were found to be in G0 or G1 phase of cell cycle while only a very low percentage of cells were in other phases of cell cycle after DSB induction for up to 9 days.
Figure 12. Possible DSB repair mechanisms at different phases of cell cycle.

During Go and G1 phase intrachromosomal recombination occurs to repair DSB by HR while sister chromatid exchange occurs during G2, M and S phase of cell cycle.
Figure 13. Assay for homologous recombinational repair of an induced DSB within a direct-repeat substrate, DR-GFP assay.
EFFECT OF ECM ON HR IN JUNCTIONED CELLS

Previously in our lab, DSB was induced in S1 DR9 cells by transfecting the I-SceI endonuclease encoding pBAS after the cells formed cell-cell junctions (approximately 40% confluence). In the presence of ECM, the percentage of GFP positive S1 DR9 cells increased indicating an up-regulation of HR as compared to its down-regulation in the absence of ECM (Figure 14). The results were also consistent with primary mouse epithelial cells with proper junctions. Furthermore, similar results obtained for non-dividing S1 DR9 cells that have normal cell-cell junctions showing that the result is not a secondary consequence of the effects of ECM on cell cycle.

EFFECT OF ECM ON HR IN SINGLE CELLS

Interestingly, single S1DR9 cells as compared to junctioned cells, ECM was found to have an opposite effect. That is, HR was up-regulated in the absence of ECM and it was down-regulated in the presence of ECM (Figure 15). Thus there were reciprocal results for the effect of ECM on HR in the presence of normal cell-cell junction and in single cells without in-vivo like cell-cell junction.

EFFECT OF ECM ON DNA REPAIR PROTEIN FOCI FORMATION AFTER IONIZING RADIATION

Foci formation kinetics of S1 cells show that the effects of ECM via β1-integrin on DNA repair proteins such as H2AX and RAD51 is modulated by ECM (Figure 16). In primary mouse mammary epithelial cell organoids, in the presence of ECM, β1-integrin and cell-cell junction, γ-H2AX levels were up regulated. But in single S1 cells (absence of cell-
cell junction), γ-H2AX and RAD51 is down-regulated in the presence of ECM and β1-integrin (Figure 16). In addition, MRE11 foci kinetics is altered by ECM in single cells (Figure 16). These results suggest that cell-cell junctions alter the effect of ECM and ionizing radiation induced foci formation. In addition γ-H2AX, RAD51 and MRE11 complex of proteins are candidates to test for nuclear modulated effect on HR.
Figure 14. Effect of ECM on HR in proliferative and non-proliferative cells. The number of GFP positive cells is directly related to HR frequency. And the GFP positive cells in the presence and absence of ECM and EGF were shown in the bar graph.
**Figure 15. Non-Tumorigenic Human Cell Line S1, Single Cells.** In case of S1DR9 cells that were stopped from proliferation immediately after seeding the cells, the cells remain single on the culture flask. The frequencies of HR for single and normal proliferating cells were compared in the presence and absence of ECM.
Figure 16. Ionising radiation induced foci kinetics of $\gamma$H2AX, MRE11 and RAD51.

Foci formation kinetics of cells after 3Gy, in the presence or absence of ECM or A2BII $\beta$1-integrin blocking antibody at 100$\mu$g/ml for S1 and Ha2/5 at 10$\mu$g/ml for mouse cells.

* $t$-test $p<0.05$ and significant.
HYPOTHESIS
Previously, ECM signaling via $\beta_1$-integrin was shown to up-regulate homologous recombination (HR) in the presence of normal cell-cell junctions whereas it down-regulated HR in the absence of junctions in single cells. This led us to ask whether cell-cell junctions modulate the effects of ECM on homologous recombination. Specifically, we hypothesize that adherens junction signaling that has intersecting signaling components with ECM signaling can modify the ECM effect on HR.
METHODS AND MATERIALS
METHODS AND MATERIALS

CELLS USED

S1 cells (normal mammary epithelial cell line) were used for western blotting and indirect immunofluorescence techniques [40, 42]. S1 DR9 cells were used for all flow cytometry analysis. Since, S1 DR9 cells contain two direct repeats of non-functional GFP genes and when a double-strand break is induced within the GFP gene at I-SceI recognition site and if the break is repaired by homologous recombination then the GFP gene becomes functional [24, 43]. Therefore, to measure the frequency of homologous recombination, I used these cells.

PLASMIDS AND siRNA USED AND THEIR TRANSFECTION

pBAS plasmid and pGFP plasmids were used for DR-GFP experiments [24]. pBAS encodes for I-SceI endonuclease. pGFP encodes GFP protein and are used as positive control as well as to measure the transfection efficiency. Both the plasmids are resistant to ampicillin. These plasmids were amplified by first transforming them into competent E. coli cells. The transformed E.coli cells were plated on LB agar containing ampicillin and incubated overnight at 37°C in order to select the transformed cells. Individual colony of cells containing the plasmid were further amplified first in 3ml and then in 250ml LB liquid medium containing ampicillin by incubating them at 37°C overnight in a shaker at 250rpm. Then the plasmids were isolated using Maxiprep (Qiagen) for transfection into mammalian cells (jetPEI transfection reagent, polyplus). siRNA against
RAD51 (siGENOME SMART pool of human RAD51, Dharmacon) was used in HR experiments. 90 µl (20 µM) of RAD51 siRNA or scramble control siRNA was transfected into S1 cells in T-75 flask using 75 µl of Dharmafect transfection reagent.

ANTIBODIES

RAD51 from Calbiochem (Cat. No. Ab-1, PC130, 1:1000 dilution) and Novus (Cat. No.13E4, 1:5000 dilution) have been used for indirect immunostaining and western blot. Ki67 from Novocastra (Cat. No. NCL-Ki67p (Ki67p-CE), 1:100 dilution) or Calbiochem (Cat No., 1:100 dilution) was used for indirect immunofluorescence. β-catenin (Cat. No. BD: 610153, BD biosciences, 1:50 dilution) and γH2AX (Clone JBW301, Millipore, Cat. No. 05-636, 1:75) were used for both indirect immunofluorescence technique. E-cadherin from Abcam (Clone MB2, Cat. No. ab8993) was used as blocking antibody and She7-78 from Axxora (Cat. No. ALX-804-203, 1:150 dilution supplemented with 10mM calcium chloride) was used for indirect immunofluorescence. NBS1 (Cat. No. BD: 611870, 1:100 dilution), RAD50 (Cat. No. BD: 611010, 1:500 dilution) and MRE11 (Cat. No. BD: 611367, 1:250 dilution) from BD transduction laboratory, α-tubulin from Sigma (Cat. No. T6074-200UL, 1:1000 dilution) and Lamin B (Santa Cruz, Cat. No. sc-6216, clone C20, 1:200 dilution) were used in western blotting.

INDIRECT IMMUNOFLUORESCENCE

To study the expression of DNA repair proteins on MB2 treated and control cells, I did indirect immunofluorescence technique. For this 40,000 S1 cells were plated on each well of a 4-well chambered slide and incubated for 3-4 days. Once the cells formed cell-
cell junctions, cells in each well were treated with MB2 at three different increasing dilutions (1, 2 and 5µg/ml) and one well was used as control (MB2 untreated). After treatment, the cells were incubated for another 48 hrs. Then the cells were subjected to indirect immunofluorescence technique. For this, the cells were washed with PBS (pH 7.4) and were treated with 0.5% triton-100 in CSK buffer (100 mM NaCl, 300 mM sucrose, 10 mM Pipes, 5 mM MgCl₂, 0.25 mM NaF) with protease inhibitor (1:1000 dilution) for 10 min on ice. Then they were washed twice using CSK buffer containing protease inhibitor for 5 min each and fixed using 4% paraformaldehyde in CSK buffer (pH 7.4) at room temperature for 20 min followed by washing thrice for 20 min with PBS/glycine. Further, the cells were blocked for 1 hour using 10% goat serum in IF buffer. Primary antibodies were mixed with the blocking solution and dispensed into the wells. Then the chambered slides were wrapped with parafilm and placed in humidified chamber at 4°C overnight to avoid drying of the antibody solution. Next day, the slides were washed thrice with IF buffer for 20 min with gentle agitation followed by secondary antibody treatment (1:400 dilution) for 1 hour in dark. Then the slides were washed thrice using IF buffer for 20 min followed by nuclear attaining using DAPI (1:10000 dilution from 10µg/ml stock solution) for 10 min. After 10 min of washing in IF buffer, coverslip was placed over the slide with Vectashield and viewed under the fluorescence microscope. Same amount of exposure time were used for each slide to view all 4 wells to compare the expression levels of DNA repair proteins in control and MB2 treated cells at different dilutions.
WESTERN BLOTTING

S1 cells grown in T-75 flasks were treated with 5 µg/ml MB2 and incubated for 48 hours. Control cells were also grown simultaneously that were not treated with MB2. These S1 cells (MB2 treated and untreated) were lysed to prepare whole cell lysate and cytosolic and nuclear extracts. In order to prepare whole cell lysate, the cells were trypsinized using 0.25% trypsin for 10 minutes followed by the addition of SBTI (soy bean trypsin inhibitor) in 10ml DMEM/F12 medium. The cells collected by centrifugation were washed with PBS and lysed by sonication in 100µl of RIPA buffer (5 M NaCl, 1 M Tris-HCl, 500 Mm EDTA, 2% SDS, 1% NP-40, 1% deoxycholate, 200 mM NaVO₄, 250 mM NAF and protease inhibitor). Then the lysed cells were centrifuged at 12,000 rpm for 10 min at 4°C. Supernatant was decanted into a separate tube and stored at -85°C. Protein estimation was done for the whole cell lysate obtained using BIORAD Dc protein estimation kit with serially diluted BSA in water as standards. Following protein quantification of whole cell lysates, they were run in SDS PAGE gel and transferred overnight onto nitrocellulose membrane at 20 mA. The transferred blot was blocked for 1 hour with 5% milk and 1% BSA in TBST (Tris buffered saline with 0.05% tween-20) and probed for a specific protein using a primary antibody in TBS/TBST buffer overnight at 4°C. Membranes were then treated with anti-mouse/rabbit secondary antibody (1:5000 dilution) for 1 hour at room temperature and viewed under LICOR imager (Odyssey v1.2 software) at a wavelength of 700nm for anti-rabbit antibodies and 800nm for anti-mouse antibodies. The bands are quantified using Odyssey LICOR infrared imaging software v1.2.
\( \gamma \)-H2AX LYSATE PREPARATION AND WESTERN BLOTTING

Cell monolayer in T-75 flask were first washed with PBS and then are scraped from the flask after spreading 500\( \mu \)l of laemilli buffer (working solution) on the surface of the flask. The working solution for laemilli buffer was prepared by mixing 4750ul Laemilli buffer (BioRad cat# 161-0737) in 250ul Hem buffer (2-mercaptoethanol from Fischer cat# 034461-100) and by finally making up the volume to 10ml with water. The scraped cells in laemilli buffer were lysed using a sonicator and heated in a 100°C water bath for 7 min. The lysate was then spun at 14,000 rpm for 5 min and stored at 20°C until loading it in the SDS PAGE gel.

The lysate was spun at 14,000 rpm for 5 min before loading them in the 12% SDS PAGE gel. 30 \( \mu \)l of sample volume loaded per well and run at 60 V for 150 min. Then the gel was transferred on to the PVDF membrane (0.2 \( \mu \)m thick pore size). The PVDF membrane was first activated in methanol for \( \sim \)1 min and washed in transfer buffer for 3-5 min before using it for transferring by western blotting method. Then after transferring overnight at 20 mA for 740 min, the blot was washed in blocking buffer containing 1ml of 1% casein in 9 ml of PBS for 50 min -1 hour. Then the blot was incubated with the primary antibody (1:1000 dilution of \( \gamma \)-H2AX from Millipore, clone JBW301, catalogue no. 16-193) in blocking buffer at 4°C overnight. Next day, the blot was washed with PBST (contains 200 \( \mu \)l Tween 20 in 1litre PBS) 4 times for 15 min each. The blot was treated with anti-mouse secondary antibody (1:5000 dilution) in the
blocking buffer for 1.40 hours and washed again with PBST 4 times for 15 min before scanning.

TOTAL AND ACTIVATED β1- INTEGRIN EXPRESSION ON CELL SURFACE

S1 cells were trypsinized and 500,000 cells were taken in 10 microcentrifuge tubes. Out of ten, one tube was used as control without primary and secondary antibody treatment, three was used as secondary control (treated just with secondary antibody and not primary antibody) to subtract the background effect due to secondary antibody, three was used for probing total β1-integrin and remaining three for probing activated β1-integrin levels in cells. The cells were first centrifuged at 8000 rpm for 5 min to remove the growth medium. Then the cell pellet was washed with 1 ml of FACS buffer (5% FBS and 0.1% sodium azide in 100 ml PBS) thrice by resuspending in FACS buffer and centrifuging at 5000 rpm for 5 min. Then the cells were treated with 50 µl of primary antibody (either total or activated β1-integrin) in FACS buffer at 1:10 dilution for 1 hour. This mixture was then re-suspended every 15-20 min up to 1 hour. The cells were washed thrice in FACS buffer by re-suspending the cell pellet in 1 ml FACS buffer and centrifuging the cells at 5000 rpm for 5 min. Then the cells were treated with 50µl of secondary antibody in FACS buffer at 1:100 dilution for 30 min. The mixture is re-suspended every 15 min during secondary antibody treatment. Later, the cells were washed with 1ml FACS buffer thrice as before. The cells were fixed by resuspending the cell pellet in 100 µl of 2% paraformaldehyde in PBS at pH 7 and stored at 4°C. The cells were suspended in 500 µl PBS before running on flow cytometer.
RESULTS
RESULTS

E-CADHERIN BLOCKING AND CONFIRMATION
Since ECM has a reciprocal effect on HR in the presence and absence of cell-cell junctions I was interested to know the effect of cell-cell junctions on DNA repair proteins. As E-cadherin is the major protein that is involved in adhesion of adjacent breast epithelial cells together I tried to find out whether this cell-cell junction protein is the one that is responsible for the reciprocal effect on HR. To do this, I used MB2 antibody. MB2 blocking antibody specifically reacts with 80 KD trypsin-resistant extracellular domain of E-cadherin that is present on the cell surface. When S1 cells at ~40% confluence are treated with 5 µg MB2 antibody per ml of H14 growth medium containing EGF and incubated for 48 hours, S1 cells were found to be scattered. The adhesion between adjacent cells appeared to be less when compared to control cells under light microscope (Figure 17). Thus, MB2 antibody causes morphological changes in S1 cells that are consistent with diminished adherens junction function. When ~40% confluent S1 cells are treated with ECM, the cells look more compact than without ECM and in case of ECM and MB2 treated cells, the scattering was found to be lesser than cells treated with MB2 in the absence of ECM (Figure 17A).

If ~40% confluent S1 cells were treated with H14 growth medium without EGF for 4 days, the cells stop from proliferating (Figure 17B). When to these non-proliferating cells MB2 antibody was added (5 µg/ml, 48 hours), cell scattering was found to be lesser than in MB2 treated normal proliferative cells. The cell morphology of ECM and ECM plus MB2 treated non-proliferative cells are also shown in Figure 17B.
To further confirm that MB2 antibody blocks E-cadherin, I seeded S1 cells in a 4-well chamber slide and incubated until the cells form cell-cell junctions. Then cells in each well were treated with MB2 antibody at different concentrations (1 µg/ml, 2 µg/ml and 5 µg/ml). After 48 hours of incubation the cells were fixed and indirect immunofluorescence was performed against E-cadherin on MB2 treated and untreated cells. Results showed that the control cells have a thin line of E-cadherin between two adjacent cells but it is absent in MB2 treated cells (Figure 18). The E-cadherin signal observed between two adjacent control S1 cells is due to the presence of homophillic interactions between E-cadherin of adjacent cells and this interaction is abolished in MB2 treated cells further confirming that MB2 blocks E-cadherin protein function at least after 48 hours (Figure 18).
Figure 17. Cell morphology of proliferative and non proliferative S1 cells with different treatments. A, changes in the morphology of proliferative cells with and/or without ECM and MB2 blocking. S1 cells were grown up to ~40% confluent by incubating for 3-4 days. ECM (10%) and/or MB2 blocking antibody (5 µg/ml) is added after the cells became 40% confluent and incubated for 48 hours. B, morphological changes in non-proliferative cells with and/or without ECM and MB2 blocking. S1 cells were grown up to ~40% confluent by incubating for 3-4 days and treated with growth medium without EGF for 4 days to stop them from proliferating. ECM (10%) and/or MB2 blocking antibody (5 µg/ml) is added after the cells became 40% confluent and incubated for 48 hours.
Figure 18. **Effect of E-cadherin blocking in S1 cells.** The Top panel, DAPI staining; Bottom panel, corresponding E-cadherin staining. 5 µg/ml of MB2 antibody is the optimal concentration used to block S1 cells.
EFFECT OF E-CADHERIN ON DNA REPAIR PROTEINS IN PROLIFERATIVE CELLS

As a next step, I was interested to find out whether E-cadherin is the cell-cell junction protein that is involved in modulating HR. For this, I tried to look at the effect of DNA repair proteins by blocking E-cadherin. Therefore, I treated the S1 cells with different concentrations of MB2 and after 48 hours of incubation the cells were fixed and indirect immunofluorescence was performed against DNA repair proteins γ-H2AX and RAD51.

In addition, proliferative marker Ki67 was examined. β-catenin (protein that binds to the cytosolic domain of E-cadherin) localization was also determined since, its movement from the cell junction to the nucleus could have down-stream consequences in DNA repair either directly or indirectly via altering transcription or signaling events.

Ki67

Ki67 stains the nucleus of viable and proliferation-proficient cells. I was able to see 22.6% (SD = +/- 0.1) positive cells in normal S1 cells (control). In MB2 treated cells (5µg/ml), 48% (SD = +/- 0.04) of cells were found to be positive (Figure 19). These results suggest that the MB2 treated cells have twice the amount of proliferative cells when compared to control cells. These results are consistent with a role for E-cadherin in reducing growth by contact inhibition and show that I blocked this E-cadherin function by MB2 treatment of S1 cells.
**β-catenin:**

β-catenin is a protein that binds to the cytoplasmic domain of E-cadherin and connects E-cadherin to the actin cytoskeleton indirectly. It is also capable of entering into the nucleus and binding to the transcription factor TCF/LEF to up-regulate genes such as cyclinD and c-myc in response to Wnt signaling. In order to determine the effect of E-cadherin blocking on β-catenin in S1 cells, we did indirect immunofluorescence. In control cells, β-catenin appeared to be around the inner side of the cell membrane attached to E-cadherin as expected but in MB2 treated cells β-catenin was more concentrated in the nucleus and was observed to be lining the inner side of the membrane attached to the cytosolic domain of E-cadherin (Figure 20). Considering cells that have β-catenin in the nucleus as positive cells, I was able see 12% (SD = +/- 0.03) positive cells in MB2 treated cells while control cells did not have any positive cells (0% SD = 0) (Figure 20).

**γ-H2AX:**

γ-H2AX is a DNA repair protein and can be seen as foci near double strand DNA breaks. Its foci formation kinetics in response to ionizing radiation was altered by ECM in opposite direction. In the presence and absence of cell-cell junctions, I determined if E-cadherin affects its expression or localization. These were done in the absence of ionizing radiation to determine if E-cadherin has effects on the basal levels of γ-H2AX. There were 17% (SD = +/- 0.08) of MB2 treated cells containing γ-H2AX foci but the
control cells did not have $\gamma$-H2AX foci in their nucleus (0%; SD = 0) (Figure 21) and this difference was significant ($p = 0.03$). These results suggest that either the basal level of DNA damage is increased or the cells have an altered basal state or concentration of DNA damage protein such as $\gamma$-H2AX in the absence of E-cadherin at cell-cell junctions. Alternatively, increased proliferation could increase replication blocks and $\gamma$-H2AX foci.

**RAD51:**

RAD51 is required for most HR and was found to have altered foci kinetics by ECM signaling. To find out if it could be modulated by cell-cell junctions, I determined the effects of E-cadherin blocking on RAD51 levels in the absence of exogenous damage.

Indirect immunofluorescence against RAD51 revealed 5% (SD = +/- 0.002) increase of RAD51 as large nuclear “foci” in MB2 treated cells when compared to control cells (0%, SD = 0) (Figure 22). The large blobs of RAD51 in the nuclei are not the typical foci observed after ionizing radiation. Whether these accumulations of RAD51 are active complexes or a means for storing inactive complexes remains an open question.
Figure 19: Ki67 expression in normal and MB2 treated S1 cells. A, Top panel, DAPI (nuclear) staining of control and MB2 treated cells respectively; Bottom panel, Ki67 staining in control and MB2 treated cells. B, The average (mean) number of Ki67 positive cells divided by the number of DAPI nuclei (y axis) in normal and in MB2 treated cells at different concentrations (1 µg/ml, 2 µg/ml and 5 µg/ml) (x axis) were represented as bars. The error bars represent standard deviation of mean (N=2), with a minimum of 2 wells and 200 cells per well counted per experiment. p-value is calculated by comparing control and MB2 treated cells at 5 µg/ml concentration.
Figure 20. Effect of E-cadherin blocking on β-catenin indirect immunofluorescence in S1 cells. A, Top panel, DAPI staining of control and MB2 treated S1 cells. Bottom panel, β-catenin staining on control and MB2 treated cells. B, the average (mean) number of positive nuclei divided by number of DAPI in control cells as y-axis and cells treated with increasing concentrations of MB2 blocking antibody as x-axis. The error bars represent standard deviation of mean (N=2), with 2 wells and minimum of 200 cells counted per well in each experiment. p-value is calculated by comparing control and MB2 treated cells at 5 µg/ml concentration.
Figure 21. Indirect immunofluorescence of γ-H2AX on control and MB2 treated cells. A, Bottom panel, γ-H2AX staining on control and MB2 treated cells and their corresponding DAPI staining in the Top panel. B, shows a graphical representation of the average (mean) number of positively stained γ-H2AX nuclei divided by number of DAPI (y-axis) against different concentration of MB2 (x-axis). The error bars represent standard deviation of mean (N=2), with 2 wells and a minimum of 200 cells counted per well in each experiment. p-value is calculated by comparing control and MB2 treated cells at 5 µg/ml concentration.
Figure 22. RAD51 expression on normal S1 cells and MB2 treated cells. A, Bottom panel, RAD51 indirect immunostaining. Top panel, DAPI staining for the control cells and MB2 treated cells. B, bar graph representing the average (mean) number of RAD51 positive nuclei divided by DAPI (y-axis) in control and cells treated with increasing concentration of MB2 treatment (x-axis). The error bars represent standard deviation of mean (N=2), 2 wells and a minimum of 200 cells per well per experiment. p-value is calculated by comparing control and MB2 treated cells at 5 µg/ml concentration.
EFFECT OF E-CADHERIN ON DNA REPAIR PROTEINS IN NON-PROLIFERATIVE CELLS

Previously, S1 cells were cultured in the presence of EGF and the effect of E-cadherin blocking was determined in dividing cells. Proliferation of MB2 treated cells was found to be higher than control cells as there was an increased Ki67 positive nucleus in MB2 treated cells than control cells. It has been found that the levels of RAD51 are cell cycle regulated being lowest in G0/G1 phase, higher in S phase and the highest in G2/M phase [44]. γ-H2AX levels have been revealed to be 3x levels higher in G1 than in S or G2/M phase of unirradiated cells [45]. So, to find out whether increase in γ-H2AX and RAD51 in MB2 treated cells is due to proliferation or MB2 blocking, I performed indirect immunofluorescence in non proliferative cells.

**Ki67:**

Ki67 expression was low in all cells due to lack of EGF in growth medium of S1 cells. In control cells there were 8% (SD = +/- 0.08) positive cells and in MB2 treated cells (5µg/ml) there were 3% (SD = +/- 0.03) positive cells (Figure 25). The p-value for control and MB2 treated cells was not found to be significant.

**β-catenin:**

In non-proliferative cells, β-catenin (Figure 24) was found to be less near the membrane than in cytosol and / or nucleus in MB2 treated cells (1.4%, SD = +/- 0.02) when
compared to control cells (0%, SD = 0). This again confirms that E-cadherin function is blocked by MB2 antibody. Here, I found that MB2 treated non-proliferating cells also increased β-catenin nuclear positive cells. However, the number of positive cells having β-catenin in the nucleus of MB2 treated cells was lesser in non-proliferative cells when compared to proliferative cells.

**γ-H2AX:**

To ask whether the MB2 induced nuclear γ-H2AX in proliferating cells is due to increased proliferation or not, I did indirect immunofluorescence with non-proliferating S1 cells in the absence of EGF. Interestingly, percent γ-H2AX foci containing nuclei were significantly higher in MB2 treated cells (15%, SD = +/- 0.2) than in control cells (0.1%, SD = +/- 0.002) in a way similar to proliferative cells (Figure 25). The number of γ-H2AX positive percent was comparable in proliferating and non-proliferating cells. Thus, this confirms that the increase in γ-H2AX levels in proliferative cells is not because of increased proliferation but as a consequence of MB2 blocking and due to a more direct effect on the DNA damage and repair pathway, including γ-H2AX phosphorylation.

When the cells were treated with medium without EGF for 6 days instead of usual 4 day treatment, the γ-H2AX foci was seen in both control (34%, SD= +/- 0.4) and MB2
treated cells (38%, SD=+/− 0.5) to a similar extent. There was no significant difference in
the γ- H2AX foci containing cell percentage between control and MB2 treated cells. But
the foci were found to be larger in MB2 treated cells than in control cells. This qualitative
observation needs to be further verified by foci size quantification in future experiments.
Figure 23. Ki67 staining in non-proliferative cells. A, Top panel, DAPI staining. Bottom panel, Ki67 staining of control and MB2 treated cells. B, a bar graph representing average (mean) Ki67 positive nuclei divided by the number of DAPI (y-axis) in control and increasing concentration of MB2 treated cells (x-axis). The error bars represent standard deviation of mean (N=2), with 2 wells and a minimum of 200 cells per well per experiment. p-value is calculated by comparing control and MB2 treated cells at 5 µg/ml concentration.
Figure 24. Effect of MB2 on the localization of β-catenin in non-proliferating S1 cells. A, Top panel, DAPI staining. Bottom panel, β-catenin staining of control and MB2 treated cells respectively. B, the difference in the average (mean) number of nuclear β-catenin positive cells divided by number of DAPI (y-axis) in control and increasing concentration of MB2 treated cells (x-axis). Positive cells are those that have β-catenin staining in the nucleus. The error bars represent standard deviation of mean (N=2), with 2 wells and minimum of 200 cells per well per experiment. p-value is calculated by comparing control and MB2 treated cells at 5 µg/ml concentration.
Figure 25. γ- H2AX expression in non-proliferating S1 cells and MB2 treated cells.

A and C, DAPI staining. Bottom panel, γ- H2AX staining of S1 cells (control) and MB2 treated cells after 48 hours. A, S1 cells that are treated with growth medium without EGF for 4 days and C, 6 days. B and D, comparison of the average (mean) number of γ- H2AX positive nuclei divided by number of DAPI (y-axis) in control and with increasing concentration or 5 µg/ml concentration of MB2 treated cells (x-axis). The error bars represent standard deviation of mean (N=2), with 2 wells and a minimum of 200 cells per well per experiment. p-value is calculated by comparing control and MB2 treated cells at 5 µg/ml concentration.
DETERMINING THE EFFECT OF E-CADHERIN ON DNA REPAIR PROTEINS BY WESTERN BLOT

To follow up on the localization results obtained by indirect immunofluorescence technique I did western blot to quantify RAD51, γ-H2AX and β-catenin in control and MB2 treated cells. In addition to this I also tried to find out whether there are any changes in the levels of NBS1, MRE11 and RAD50 in control and MB2 treated cells since, previously the MRE11 foci formation in response to ionizing radiation has also shown to be altered by ECM. As a first step, I prepared whole cells lysates from control and MB2 treated cells. For this I grew S1 cells up to ~40% confluence and treated them with MB2 blocking antibody (5 µg/ml). After 48 hours the cells were trypsinized and lysed. The whole cell lysates of normal S1 control cells and MB2 treated cells were run on an SDS PAGE and were examined for the presence of β-catenin (Figure 26), RAD51 and γ-H2AX (Figure 27) and MRE11/RAD50/NBS1 (Figure 28).

Results show no significant difference in the total β-catenin levels between the control and Mb2 treated cells. But both in proliferative and non-proliferative cells, nuclear β-catenin levels were found to be significantly higher than that of cytoplasmic extract in MB2 treated cells when compared to normal S1 cells (Figure 26). These results confirm the altered β-catenin localization in MB2 treated cells shown by indirect immunofluorescence method.
Total RAD51 levels were found to be significantly lower (Figure 27 A and B) in proliferative MB2 treated cells when compared to control cells (P = 0.04). Even though indirect immunofluorescence results showed an increased RAD51 “foci” in MB2 treated cells than control, the total RAD51 levels in MB2 treated cells were lower than in control. This suggested that the localization of the RAD51 is regulated independent of the protein levels overall. Whether the nuclear blobs of RAD51 represent active complex ready to repair or inactive complexes that need to be activated are two possibilities to be explored further.

Furthermore, total γ-H2AX was found to be significantly higher in MB2 treated proliferative cells than untreated cells and results also showed that γ-H2AX levels were also found to be fluctuating in non-proliferative cells (Figure 27 C). The bands were quantified and normalized with α-tubulin loading control. The up-regulation of γ-H2AX in proliferating cells was more consistent than in non-proliferating cells. This may be possibly aided by the positive effect of MB2 on proliferation that increases γ-H2AX in S and G2/M phases of the cell cycle.

There is no significant difference in the amount of MRE11, RAD50 and NBS1 proteins in control and MB2 treated cells either under proliferating or non-proliferating conditions (Figure 28).
Figure 26. β-catenin levels in MB2 treated and untreated cells. β-catenin levels in whole cell, cytosol and nucleus of proliferative cells (A, B) and non-proliferative cells (C, D). α-tubulin is used as control for whole cell lysate and cytosol extract, lamin B is used as control for nuclear extracts. B, the nuclear-to-cytosolic ratio in control and MB2 treated cells. The error bars represent standard deviation of mean (N=3). D, a nuclear-cytosolic ratio of β-catenin in MB2 treated cells when compared to control cells. The error bars represent standard deviation of mean.
(N=2). p-value is calculated for the bar graphs by comparing control and MB2 treated cells at 5 µg/ml concentration.

**Figure 27. RAD51 and γ- H2AX levels in control and MB2 treated cells.** A, western blot probed for total RAD51 levels in control and MB2 treated cells. α-tubulin was used as a loading control. B, total RAD51 levels normalized by α-tubulin in control and MB2 treated cells represented as a bar graph. RAD51/ α-tubulin was used a y-axis. The error bars represent standard deviation of mean (N=3). p-value is calculated by comparing control and MB2 treated cells at 5 µg/ml concentration. C, a sample blot containing normal and MB2 treated whole cell lysate from proliferative and non-proliferative cells probed for γ- H2AX levels. D, α-tubulin normalized γ- H2AX levels in control and MB2 treated cells. The error bars represent standard deviation of mean (N=2). p-value is calculated by comparing control and MB2 treated cells at 5 µg/ml concentration.
Figure 28. MRN complex of proteins in control and MB2 treated proliferative cells.

A, a representative western blot of control and MB2 treated lysates collected from proliferative and non-proliferative S1 cells. The lysates were probed for RAD50, NBS1 and MRE11 and α-tubulin was used as loading control. B, C and D, levels of NBS1, RAD50 and MRE11 respectively in control and MB2 treated proliferative (+EGF) and non-proliferative (-EGF) cells. The error bars represent standard deviation of mean (N=4 for proliferative cells and N=3 for non-proliferative cells). p-value is calculated by comparing control and MB2 treated cells at 5 µg/ml concentration.
EFFECTS OF E-CADHERIN ON β1-INTEGRIN EXPRESSION AND ACTIVATION

Previously, β1-integrin blocking in single cells was shown to abolish the ECM effect on HR, as well as on foci formation in response to ionizing radiation. Combined with data in the literature that E-cadherin pathway can alter integrin expression and activation I wanted to determine the effect of E-cadherin blocking on cell surface β1-integrin expression and activation by immunostaining non-permeabilized cells, as well as the total protein levels by western blot.

β1-integrin cell surface expression in control and MB2 treated cells was not significantly different either in proliferating or non-proliferating conditions (Figure 29 A, B, C and D). The total levels of β1-integrin in control and MB2 treated proliferative and non-proliferative cells measured by western blot also showed no significant difference (Figure 29 E and F), showing that E-cadherin has no effect on β1-integrin expression, localization, or activation.
Figure 29. Effect of E-cadherin blocking on the total and activated β1-integrin expression levels. Both in proliferative (A and B), and non-proliferative (D and E), normal S1 cells and MB2 treated cells were immunostained for cell surface expression levels of total and activated β1-integrin. The error bars represent standard deviation of mean (N=2 for both proliferative cells and non-proliferative cells with 3 samples for each condition per experiment). p-value is calculated by comparing control and MB2 treated cells at 5 µg/ml concentration. C and F, α-tubulin normalised total levels of β1-integrin in control and MB2 treated proliferative and non-proliferative cells respectively. The error bars represent standard deviation of mean (N=2 for both proliferative cells and non-proliferative cells). p-value is calculated by comparing control and MB2 treated cells at 5 µg/ml concentration.
INTERACTION OF E-CADHERIN WITH ECM AND HR

Previously, ECM was shown to up-regulate HR in junctioned cells but down regulate it in single cells via β1-integrin. My protein localization and expression data showed that E-cadherin prevents repair proteins RAD51 and γ-H2AX from entering into the nucleus, but does not alter β1-integrin levels. These data prompted the question of whether E-cadherin was involved in mediating HR or the effect of ECM on HR. Therefore, DR-GFP assay was conducted in S1 DR9 cells. S1 DR9 cells are those that have two direct repeats of non-functional GFP genes. When one of the GFP genes is cut with I-SceI endonuclease (encoded by pBAS), it causes a double strand break. If this break is repaired by HR, then the GFP gene becomes functional and the cells can be identified and measured by flow cytometry due to the expression of reporter gene in them. GFP gene remains non-functional if they are repaired by non-homologous end joining. Using this assay the HR frequency was measured.

To identify whether there is any interaction between E-cadherin, ECM and HR, the DR-GFP assay was performed under 4 different conditions. The S1 DR9 cells were grown up to ~40% confluence and the cells are transfected with pBAS to introduce double strand break in the presence and / or absence of ECM and E-cadherin (namely - MB2 & - ECM, + MB2 & - ECM, - MB2 & + ECM and + MB2 & + ECM). 4 - 8 days after the introduction of DNA break, the HR frequency was measured as GFP positive cells under 4 different conditions using flow cytometric analysis. pGFP encoding GFP gene was used as transfection control.
The HR frequency was found to be higher in MB2 treated when compared to control cells in the absence of ECM while in the presence of ECM, MB2 abolishes the effect of ECM on HR (Figure 30). These results suggest that E-cadherin is necessary for the up-regulation effect of ECM on HR.
Figure 30. Frequency of HR in the presence and absence of MB2 and ECM. A, HR frequency in control and MB2 treated cells without ECM. B, HR frequency in control and MB2 treated cells in the presence of ECM. The error bars represent standard deviation of mean (N=2) with a minimum of 3 samples per condition in each experiment. p-value is calculated by comparing control and MB2 treated cells at 5 μg/ml concentration.
INTERACTION OF RAD51, ECM AND HR

My results showed that E-cadherin is necessary for mediating the up-regulation effect of ECM on HR, as well as showing that E-cadherin increases the nuclear accumulation of RAD51. This begged the question of whether RAD51 is necessary for the ECM effect on HR. Therefore, as a first step to find out the effect of HR and ECM in the absence of RAD51, siRNA against RAD51 was used. To identify, the duration during which RAD51 is down regulated by siRNA I transfected RAD51 siRNA and scramble control, 24 hours after plating 300,000 cells in a 6-well chamber plate.

Lysates were collected from cells transfected with scramble control and RAD51 siRNA on day 1, 2, 4 and 7 after transfection. Protein concentration in the lysates was measured using BioRad protein determination kit. RAD51 levels in those lysates were later measured by performing western blot technique and normalized using their respective α-tubulin levels and later by scramble controls. RAD51 siRNA was found to be down regulated during day 1 to ~ day 4 (Figure 31).

Therefore, I planned to introduce double strand break during day1 after RAD51 siRNA transfection and measure GFP positive cells to find the effects of HR in the absence of RAD51. For this, I plated S1 DR9 cells on T75 flasks. After 28 hours, the cells were transfected with RAD51 siRNA and scramble control. Then, the cells were transfected with pBAS to induce double strand break on the I-SceI recognition site within the non-functional GFP gene 18-19 hours after RAD51 siRNA transfection during which RAD51 was down regulated. To find out the effect of ECM and HR in the presence and absence
of RAD51, double strand break was induced under 4 different conditions namely, in the absence of ECM and presence of RAD51, in the absence of ECM and RAD51, in the presence of ECM and RAD51 and in the presence of ECM and absence of RAD51. GFP positive cells were measured 48 hours after pBAS transfection that induces double strand break, to measure the frequency of HR under 4 different conditions mentioned above (to ensure the experiment was completed within the time frame of RAD51 down-regulation). Plasmid encoding GFP protein is also transfected for all 4 growth conditions to measure the transfection efficiency under each condition and also to normalize the results.

In the absence of ECM, HR was significantly down regulated in RAD51 siRNA transfected cells ~9-fold than control cells. While in the presence of ECM, the HR frequency is not significantly down regulated in the presence of RAD51 siRNA (Figure 32). These results could suggest that ECM induces a largely RAD51-independent pathway of HR, if the siRNA down-regulation of RAD51 was succesful in the presence of ECM as in its absence. To determine this, lysates were collected from cells that were treated with scramble siRNA and RAD51 siRNA in the presence and absence of ECM. When the lysates were probed for RAD51 on a western blot, I was able to see equal down regulation of RAD51 in the RAD51 siRNA treated cells both in the presence and absence of ECM (Figure 33).
Figure 31. Down regulation of RAD51 using siRNA. A, western blot containing lysates prepared from RAD51 siRNA or scramble control siRNA transfected cells. The lysates were collected after 1, 2, 4 and 7 days after siRNA transfection. B, RAD51 levels were quantified from the western blot were shown in the form of bar graph. The RAD51 levels were normalized using their respective scramble control's RAD51 levels.
Figure 32. Effect of RAD51 siRNA on HR in the presence and absence of ECM.

The figure shows the HR frequency in RAD51 siRNA and scramble control transfected cells in the presence and absence of ECM. The error bars represent standard deviation of mean (N=2), a minimum of 3 samples per condition was used in each experiment. p-value is calculated by comparing HR frequency of scramble control and RAD51 siRNA transfected cells in the presence or absence of ECM.
Figure 33. RAD51 levels in scramble and RAD51 siRNA transfected cells. Western blot shows the levels of RAD51 in the lysates of scramble siRNA and RAD51 siRNA transfected S1 cells in the presence and absence of ECM. α-tubulin was used as the loading control.
β-CATENIN’S ROLE IN HR AND ITS INTERACTION WITH ECM

Since I was able to see in indirect immunofluorescence and western blot that the β-catenin levels are higher in the nucleus of MB2 treated cells I was interested in finding out whether there was any change in the phosphorylated forms of β-catenin. For this I probed for phosphorylated forms of β-catenin such as β-catenin Tyr-142, Ser-45 and tyr89. I was not able to see any bands in case of phosphorylated β-catenin Ser-45 and tyr89. But I was able to see a band for phosphorylated β-catenin Tyr-142. There was no difference in the signal for phosphorylated β-catenin Tyr-142 in control and MB2 treated proliferative and non-proliferative cells (Figure 34).
Figure 34. **Tyr-142 phosphorylated β-catenin levels in MB2 treated and untreated cells.** The blot shows phosphorylated β-catenin Tyr-142 levels in proliferative control and MB2 treated cells within lane 1 and 2. Lane 3 and 4 has lysates from non-proliferative control and MB2 treated cells. α-tubulin was used as the loading control. The table below shows the quantification of α-tubulin normalized Tyr-142 phosphorylated β-catenin levels on the blot.

<table>
<thead>
<tr>
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<th>+ EGF</th>
<th>- EGF</th>
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<tbody>
<tr>
<td>control</td>
<td>0.1019</td>
<td>0.1212</td>
</tr>
<tr>
<td>Treated</td>
<td>0.1031</td>
<td>0.0978</td>
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DISCUSSION
DISCUSSION

Previously our lab showed that in the presence of ECM and cell-cell junctions ECM signaling up-regulated HR whereas in the absence of cell-cell junction ECM signaling was found to down regulate HR. Since, in the presence and absence of cell-cell junctions ECM signaling effects on HR changes, I was interested in identifying cell junction protein(s) that is responsible for interfering with the ECM signaling on HR.

In mammary epithelial cells, E-cadherin is the main adherens junction protein that is involved in attaching the adjacent cells together. Reduction in E-cadherin expression has been found in tumor cells [46]. It has also been reported that inactivating mutations of E-cadherin gene are highly frequent in infiltrating lobular breast carcinomas and diffuse gastric carcinomas [47]. E-cadherin is the second most frequently mutated gene in breast cancer [48].

Therefore, to see whether this cancer relevant protein, E-cadherin is the cell-cell junction protein that is interacting with HR, I blocked E-cadherin protein function of S1 cells and asked if this altered DNA repair protein localization, expression and HR function. E-cadherin blocking in the non-tumorigenic S1 cell lines resulted in cell scattering, loss of contact inhibition, increased proliferation as well as movement of β-catenin into the nucleus.

It is interesting to note that although S1 cells are non-transformed there are other examples of non-transformed cells responding similarly to E-cadherin blocking. For example, in ES cells it has been shown that loss of E-cadherin was found to result in
nuclear localization of β-catenin [49]. These results suggested that S1 cells are appropriate for studying loss of E-cadherin function and its downstream effector β-catenin.

Blocking E-cadherin in both proliferating and non-proliferating S1 cells increased γ-H2AX foci in the nucleus. Large nuclear foci of RAD51 were present in E-cadherin blocked cells but not in normal S1 cells. These results suggested that E-cadherin blocks the nuclear localization of γ-H2AX and RAD51. This could indicate that proper E-cadherin containing cell-cell junctions are important in preventing endogenous DNA damage. When E-cadherin is blocked, cell scattering could upset cellular homeostasis resulting in increased endogenous DNA damage. Reactive oxygen species are one potential DNA damage-causing agent that could be induced [50]. Increased DNA replication and stalled forks could be another way of inducing DNA repair foci in the absence of exogenous damage. Since our results showed that E-cadherin blocking increases γ-H2AX foci even in the absence of proliferation it is not necessary to invoke the S phase replication blocks in explaining the nuclear accumulation of these DNA repair proteins.

It is interesting to note that in the absence of added ECM, blocking E-cadherin up-regulated HR as well as increased the nuclear γ-H2AX and RAD51. These results were consistent with E-cadherin mediated junction signaling modulating HR via γ-H2AX and
RAD51. In fact, in the absence of ECM we found HR to be almost completely RAD51 dependent. In exact opposition to these results, we found both E-cadherin and RAD51 to act very differently for HR in the presence of ECM. RAD51 was not necessary for most HR in the presence of ECM but E-cadherin was entirely required i.e, E-cadherin prevents HR in the absence of ECM but it is needed for HR in the presence of ECM.

In trying to decipher the E-cadherin pathway components in the HR regulation, it is noteworthy to mention that our results revealed an interaction between E-cadherin, an adherens junction protein, HR proteins and movement of β-catenin into nucleus. Various studies have shown that β-catenin interacts with LEF/TCF transcription factors in the nucleus [28]. So, transcriptional regulation of DNA repair related proteins could contribute to the observed HR effects. Alternatively, β-catenin could signal via multiple phosphorylation events to cytoplasmic signaling components. Of these Tyr-142, Tyr-86 and Ser-45 have been studied well.

Phosphorylation at Tyr-142 abolishes the interaction between β-catenin and alpha-catenin. pp60c-src phosphorylates β-catenin at Tyr-86 and Tyr-654 reducing its affinity for E-cadherin [39]. Phosphorylation at Ser-45 by an unknown kinase is required for the GSK-3 to further phosphorylate β-catenin at Thr-41, Ser-37 and Ser-33 that results in beta-catenin ubiquitination and proteasome-dependent degradation [40].

Finally to find out whether there is any change in the amount of phosphorylated β-catenin levels in the normal S1 cells and MB2 treated cells, I probed for phosphorylated β-catenin Tyr142, Ser45 and Tyr86 by western blot analysis. I was not able to see any
difference in the amount of phosphorylated β-catenin Tyr142 levels between control and MB2 treated cells. Moreover, the levels of phosphorylated β-catenin Tyr86 and Ser45 proteins remained undetectable in control and MB2 treated cells. Thus I was not able to see any change in the three phosphorylated forms of β-catenin in control and MB2 treated cells.

Apart from these phosphorylated forms of β-catenin, there are also other mutant forms (especially at the N-terminal end) of β-catenin that are associated with cancer and have the ability to enter into the nucleus. For example, β-catenin that lacks Ser 45 (3bp deletion) is a mutant that is not affected by APC leading to an increase in β-catenin-LEF4 complex formation in the nucleus. This mutant is seen in colon cancer tumor cell line HCT116. Another mutant seen in SW48 colon cancer tumor cell line has a single missense mutation that replaces Ser to Tyr. This mutant is also not affected by APC leading to an increase in β-catenin-LEF4 complex formation in the nucleus. There are other N-terminal truncated β-catenin mutants that do not have GSK-3beta phosphorylation sites on β-catenin. Therefore testing these mutants for their effect on HR could also give some insights into the role of β-catenin in HR. HR experiments related to β-catenin mutants or siRNA against β-catenin and E-cadherin will help us to understand further the role of E-cadherin and β-catenin in DNA double strand break repair directly, as well as in combination with ECM signaling.

For ECM signaling and E-cadherin cross talk we observed here, the primary candidate protein was β1-integrin. However, we found that blocking E-cadherin did not alter this
integrin, making it less likely the mediator of ECM to E-cadherin cross talk in modulating HR. However, experiments blocking β1-integrin need to be performed to determine if it is functionally relevant for the E-cadherin dependence of the ECM effect on HR.

These results leave us with a model (Figure 35) indicating that ECM can act as a genome stability enhancer via inducing HR. For this, intact cell-cell junctions are needed and E-cadherin is necessary for such ECM mediated genome stability. Given that both proper cell-ECM interactions and proper cell-cell interactions are needed for tissue integrity, for cells requiring adhesion for cell survival, our results indicate that both adhesion pathways are needed for proper DNA repair pathway choices and thus maintenance of a stable genome. Based on our model, we predict that in cancer cells with either compromised cell-ECM or cell-cell junctions HR will be down regulated resulting in instability.

In conclusion, E-cadherin was found to interact with DNA repair proteins and this could occur through ECM signaling pathway since, HR experiments have shown that there is a cross-talk between E-cadherin and ECM. And this cross talk could be via β-integrin other than β1 type of integrin. Thus E-cadherin was found to have a new role in DNA repair apart from its role in cancer. Moreover, HR occurs in a RAD51-independent manner in the presence of ECM and E-cadherin would interact with this pathway as in the absence of E-cadherin, HR is down regulated in the presence of ECM. Apart from this I believe that other than E-cadherin, β-catenin should also be involved in this HR pathway directly or indirectly since I was able to see changes in the nuclear β-catenin levels in MB2 blocked cells. Further investigation on the various nuclear forms of β-
catenin (mutants/phosphorylated forms) in HR will help us to understand its involvement in the E-cadherin role on HR. Finally, understanding the inter-relationship between E-cadherin, genomic stability and β-catenin will contribute in further understanding normal breast cell processes, how they get transformed and contribute later on cancer progression.
Figure 35. Model for adherens signaling effects on HR and genome stability. 1, In the presence of both ECM and cell-cell junctions, ECM up-regulates HR. This requires E-cadherin and HR occurs in a RAD51-independent manner - stable genome expected. 2, In the absence of cell-cell junction (example: single cells), ECM blocks HR as well as downregulating RAD51 and γ-H2AX foci– unstable genome expected. 3, In the absence of ECM, E-cadherin blocks HR and nuclear RAD51 and γ-H2AX – unstable genome.
1. **CELL-ECM and CELL-CELL adhesion intact**

2. **CELL-CELL adhesion aberrant**

3. **CELL-ECM adhesion aberrant**
REFERENCES


6. Nakano, S., et al., *Differential tissular expression and localization of type IV collagen alpha1(IV), alpha2(IV), alpha5(IV), and alpha6(IV) chains and their


