Role of XRCC3 in Acquisition and Maintenance of Invasiveness through Extracellular Matrix in Breast Cancer Progression

Siddharth Saini
Virginia Commonwealth University

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Role of XRCC3 in Acquisition and Maintenance of Invasiveness through Extracellular Matrix in Breast Cancer Progression

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)

Siddharth Saini
Department of Radiation Oncology
Virginia Commonwealth University
Richmond, Virginia
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Abstract:

Acquisition of invasiveness through extracellular matrix is a crucial characteristic of transition to malignancy in the breast. It was previously shown that Polo-like kinase 1 (PLK-1), a mitotic kinase and genome stability regulator, is involved in acquisition of invasiveness in a breast cell model (HMT-3522 cell line) of pre-invasive to invasive transition. This and other data led to the suggestion that a new class of genes called GISEM for Genome Instability and Extracellular Matrix Invasiveness may exist. Previous lab data show that XRCC3 is found downregulated in progression from preinvasive to invasive phenotype. This led to the hypothesis that XRCC3 may be a negative regulator of invasion. To support this hypothesis, overexpression of XRCC3 in the invasive T4-2 cells downregulated invasion, but also growth. In order to verify the role of XRCC3 in invasiveness, and determine whether it is independent from any effects on growth, we tested the effect of downregulating XRCC3 on the invasiveness of T4-2 cells. Short-term downregulation of XRCC3 using siRNAs produced a significant increase in invasiveness, suggesting a role for XRCC3 as a negative regulator of invasion. During the invasion assay time course, XRCC3 downregulation had no effect on growth or apoptosis supporting the idea that this is a direct effect on invasion and not an artifact of the assay.

XRCC3 is one amongst the five members of the RAD51 paralog family, consisting of accessory proteins or RAD51 cofactors (namely RAD51B, RAD51C, RAD51D, XRCC2 and XRCC3) which interact with each other to form complexes (BCDX2, BC, DX2 and CX3) that collaboratively assist RAD51 in homologous recombinational repair (HRR) of
DNA double-strand breaks. To see if these interactions are important in terms of invasion, as they have been demonstrated for DNA repair, we studied the effect of XRCC3 downregulation on the levels of RAD51 paralogs. We found lowered levels of RAD51C, but not RAD51B or RAD51D, when XRCC3 was downregulated. Since XRCC3 forms the CX3 complex with RAD51C, we downregulated RAD51C using siRNAs in T4-2 cells and found this to significantly increase invasiveness. Consistent with previous findings by other groups, downregulating RAD51C also lead to decreased levels of XRCC3 in invasive T4-2 cells. These results suggest that the XRCC3-RAD51C interaction is important for invasion as well as the previously studied DNA repair function.

In delineating the mechanism by which XRCC3 acts as a negative regulator of invasion, we further questioned if XRCC3 alters secreted factors that are important for the invasiveness of T4-2 cells and tested the effects of conditioned medium (CM) from XRCC3 altered T4-2 cells on parental T4-2 cells' ability to invade. Results show a significant increase in invading T4-2 cells when suspended in CM from XRCC3 siRNA transfected T4-2 cells, suggesting a direct effect of XRCC3 siRNAs on the ability of T4-2 CM to induce invasiveness in T4-2 cells. Furthermore, we investigated the effects of XRCC3 inhibition on cell surface integrins and focal adhesion kinase (FAK). Indirect immunofluorescence results show increased formation of focal adhesions containing two phosphorylated FAK residues- autophosphorylated FAK-Y397 and FAK-Y861 (previously implicated in increased migration and invasion of tumor cells) in XRCC3 siRNA transfected T4-2 cells.
Overall, these results support a new role of XRCC3 in invasion, in addition to its previously reported role in DNA repair. These findings imply that loss of XRCC3 function in cancer progression would upregulate invasion as well as downregulate DNA repair and genome stability. Therefore, stabilization of XRCC3 function could provide a promising therapeutic against breast cancer progression. The dual role of XRCC3 in invasion and DNA repair also renders it an attractive candidate risk biomarker of breast pre-cancer to invasive cancer progression.
# TABLE OF CONTENTS

1. **Introduction**  
   1.1: XRCC3 and DNA repair  
   1.2: Polymorphisms in XRCC3 and breast cancer  
   1.3: XRCC3, apoptosis and cell cycle  

2. **Hypothesis**  

3. **Materials and Methods**  

4. **Results**  
   4.1: Human breast cell line model of pre-invasive to invasive transition  
   4.2: Rationale for testing XRCC3: XRCC3 expression alterations in progression  
   4.3: XRCC3 overexpression effects on invasion and growth  
   4.4: Effect of siRNA downregulation of XRCC3 on acquisition and maintenance of invasiveness  
   4.5: Effect of XRCC3 siRNA on the Boyden chamber/ transwell invasion assay based invasiveness of T4-2 cells  
   4.6: Cell proliferation assay for the effect of XRCC3 siRNA on T4-2 cells  
   4.7: TUNEL apoptosis assay for the effect of XRCC3 siRNA on T4-2 cells  
   4.8: Effect of XRCC3 siRNAs on RAD51 paralog levels  
   4.9: Downregulation of RAD51C and its effect on invasion  
   4.10: Effect of RAD51C downregulation on XRCC3 protein levels  
   4.11: Effect of XRCC3 siRNAs on the ability of T4-2 conditioned medium to induce invasiveness in T4-2 cells  
   4.12: Effect of XRCC3 siRNA on activation and localization of FAK  
   4.13: Effect of XRCC3 siRNAs on cell-surface beta-1 integrin levels  

5. **Discussion**  

6. **References**
# LIST OF FIGURES

**Figure 4.1:** HMT3522 series of cell lines  
**Figure 4.2:** Western blot for XRCC3 protein  
**Figure 4.3:** Western blot showing overexpression of XRCC3  
**Figure 4.4:** Phase contrast images of transduced T4-2 cells  
**Figure 4.5:** Effect of XRCC3 overexpression on invasion  
**Figure 4.6:** siRNA downregulation of XRCC3  
**Figure 4.7:** Effect of transient downregulation of XRCC3 using siRNAs on invasiveness of T4-2 through laminin-rich basement membrane (lrBM) in transwell invasion assays  
**Figure 4.8:** Optimizing MTT for T4-2 cells  
**Figure 4.9:** Effect of XRCC3 siRNA on T4-2 cell proliferation / growth  
**Figure 4.10:** Effect of XRCC3 siRNAs on apoptosis  
**Figure 4.11:** Effect of XRCC3 siRNA on RAD51 paralog levels  
**Figure 4.12:** siRNA mediated downregulation of RAD51C and its effects on invasion  
**Figure 4.13:** Effect of RAD51C downregulation on XRCC3 protein levels  
**Figure 4.14:** Effect of XRCC3 siRNA on the ability of T4-2 conditioned medium to induce invasiveness in T4-2 cells (secreted factors)  
**Figure 4.15:** Effect of XRCC3 siRNA on expression and localization of total FAK  
**Figure 4.16:** Panel of total FAK controls  
**Figure 4.17:** Effect of XRCC3 siRNA on total- FAK protein levels  
**Figure 4.18:** Effect of XRCC3 siRNA on expression & localization of autophosphorylated FAK-Y397  
**Figure 4.19:** FAK-Y397 expression in the presence of growth factors  
**Figure 4.20:** Effect of XRCC3 siRNA on FAK-Y397 protein levels  
**Figure 4.21:** Effect of XRCC3 siRNA on expression & localization of FAK-861  
**Figure 4.22:** Panel of FAK-Y861 controls  
**Figure 4.23:** Effect of XRCC3 downregulation on cell-surface beta-1 integrin levels  
**Figure 5.1:** Model for XRCC3 as a negative regulator of invasion
1. Introduction:

The extracellular matrix (ECM) regulates a cell's dynamic behavior. Cells bind to components of the extracellular matrix. This cell-to-ECM adhesion is regulated mainly by specific cell surface cellular adhesion molecules known as integrins\(^1,2\). Integrins are heterodimeric cell surface adhesion receptors composed of α- and β-subunits, which mediate cell-extracellular matrix (ECM) and cell-cell interactions. Integrins not only send signals to the cell in response to the extracellular environment, but they also respond to intracellular cues and alter the way they interact with the extracellular environment\(^1,3\).

As cancer cells become invasive, they develop altered affinity for their extracellular matrix (ECM). Changes in the phenotype are mediated by alterations in the expression of integrins, release of metalloproteases that remodel the ECM, and the deposition of new ECM molecules. These activate signalling cascades that regulate gene expression, cytoskeletal organization, cell adhesion, cell survival, cell migration and invasion.

A) Secreted Factors: Amongst the proteolytic enzymes involved in invasion are matrix-degrading enzymes like matrix-metalloproteinases (MMPs) that allow cancer cells to proteolize, invade the basement membrane ECM and metastasize. In addition to tumor cells themselves, secreted MMPs like MMP2 and MMP9 have been observed to be upregulated in stromal cells and deposited on the tumor microenvironment, contributing to tumor progression\(^4\). In terms of acquisition of invasiveness in the human breast cancer model of pre-invasive to invasive transition that we use here (HMT3522 cell line)\(^5\), microarray analysis has shown elevated expression of MMP9, MMP13, MMP15 and MMP17 in invasive cells as compared to their pre-invasive counterparts.
Flow cytometric analysis showed higher cell-surface levels of MMP15 and MMP17 in invasive cells as compared to pre-invasive cells. Since siRNA downregulation of these MMPs reduce invasiveness, they are implicated in invasive behavior of these breast epithelial cells. Consistent with this, the conditioned medium from invasive cells overexpressing these MMPs could induce invasiveness in pre-invasive breast cells.

B) **Integrin-mediated signaling:** Central to the many roles that integrins play in cancer are integrin-mediated signal transduction processes\(^\text{6, 7}\). These act by regulating kinases like focal adhesion kinase (FAK) and Src kinase family members. Activation of the FAK-Src complex is central to regulation of downstream signaling pathways that control cell spreading, cell movement, cell survival, cell migration and invasion by mediating integrin- or serum-induced activation of downstream pathways like the RAS–extracellular-signal-regulated kinase (ERK) pathway or the JNK/NFκB pathways\(^\text{8,9}\). FAK is expressed at higher levels in some invasive tumors than in benign pre-neoplastic tumours\(^\text{9}\).

In addition to these invasion mechanisms, we found one DNA damage repair gene, Polo-like kinase 1 (PLK1) as a result of gene expression analysis comparing pre-invasive cells to invasive cells\(^\text{10}\). PLK1 expression was found to increase in many invasive carcinomas. Downregulation of PLK1 using siRNA in invasive cells significantly decreased invasion, making PLK1 a positive regulator of acquisition and maintenance of invasion. Furthermore, it was found that PLK1 regulates invasion via a mechanism of phosphorylating vimentin which in turn controls the cell surface levels of β1 integrin. Using β1-integrin blocking antibody, and siRNA against vimentin, it was found that
exclusive inhibition of both resulted in a decrease in invasiveness in invasive cells. Moreover, the effects of combined downregulation of β1-integrin, vimentin and PLK1 were not additive, suggesting that the three proteins may function in the same invasion pathway. In addition, Ku80, another protein involved in genome stability via double-strand break repair has been shown to interact with MMP9 at the cell surface, remodelling the ECM with the deposition of new ECM molecules and accounting for invasiveness in an immune cell system.

These results led to the suggestion that a new class of genes called GISEM for Genome Instability and Extracellular Matrix Invasiveness may exist. Additional candidate proteins for GISEM genes were found by comparing gene expression profiles of pre-invasive and invasive cell counterparts of the HMT-3522 series. XRCC3 which is a protein involved in DNA double-strand break repair was found to be downregulated in progression. This project follows up on these findings by examining the role of this genome stability related gene XRCC3 in invasion.

### 1.1 XRCC3 and DNA repair:

XRCC3 (14q32.3) is one of a group of genes originally identified by their ability to complement certain mutated rodent cell lines for hypersensitivity to ionizing radiation and other DNA damaging agents, and hence designated “X-ray repair cross-complementing”12. XRCC3 was originally identified as a gene able to complement the DNA damage sensitivity, chromosomal instability and impaired growth of the mutant hamster cell line irs1SF13. XRCC3 possesses a limited sequence similarity to Rad51.
and interacts with it. Rad51 is a member of a family of eukaryotic proteins related to the bacterial recombinational repair protein RecA\(^1\), required for homologous recombination. Homology-directed recombinational repair of DNA damage has recently emerged as a major mechanism for the maintenance of genomic integrity in mammalian cells as well\(^2\). The highly conserved strand transferase, Rad51, is critical for this process. Rad51 plays a central role in homologous DNA recombination, and its activity is controlled by a number of Rad51 cofactors. These include five Rad51 paralogs-Rad51B, Rad51C, Rad51D, XRCC2 and XRCC3\(^3\). Error-free homology-directed repair of DNA double-strand breaks is decreased 25-fold in an XRCC3-deficient hamster cell line and can be restored to wild-type levels through XRCC3 expression\(^4\).

It was previously known that all five RAD51 paralogs participate collaboratively in DNA repair. However, this idea was challenged by the biochemical identification of two independent complexes composed of either Rad51B/C/D/XRCC2 or Rad51C/XRCC3\(^5\). Colony survival assays involving double mutant knockouts suggested that human Rad51 paralogs form several different complexes in the cells. These have been described as BCDX2 complex containing Rad51B–Rad51C–Rad51D–XRCC2, the BC sub-complex containing Rad51B–Rad51C, the DX2 sub-complex containing Rad51D–XRCC2 and the CX3 complex Rad51C–XRCC3\(^6\). The BC and DX2 sub-complexes bound to single stranded DNA and double-stranded DNA, and hydrolyzed ATP, and BC sub-complex supports the strand exchange reaction mediated by the Rad51 and RPA proteins. The CX3 (RAD51C–XRCC3) complex binds ssDNA, but not duplex DNA, to form protein–DNA networks. The specificity of these interactions may be important for the initiation of recombinational repair as DSBs are resected to expose single-stranded
tails\textsuperscript{17}. XRCC3 protein levels correlate with RAD51C protein levels. An increase in XRCC3 protein levels has specifically shown to increase RAD51C protein levels suggesting that XRCC3 overexpression stabilizes RAD51C by the increased formation of the XRCC3-RAD51C heterodimer\textsuperscript{17}. Using siRNA against RAD51C in a human fibrosarcoma cell line HT1080 and cervical carcinoma cell line HeLa, depletion of Rad51C showed a significant reduction of frequency in homologous recombination and a sharp reduction in the levels of XRCC3 protein in Rad51C-depleted cells, suggesting that XRCC3 is dependent for its stability upon heterodimerization with Rad51C\textsuperscript{26}. The relevance of these biochemical and repair-related functional interactions of XRCC3 with RAD51C to other roles of XRCC3 remain open areas for investigation.

\textbf{1.2. Polymorphisms in XRCC3 and breast cancer:}

Polymorphisms in DNA repair genes, including double-strand break (DSB) repair genes, are postulated to confer increased cancer risk\textsuperscript{27}. Polymorphisms in genes involved in homologous recombination (NBS1, RAD52, RAD51, XRCC2 and XRCC3) and non-homologous end-joining (KU70/80 and LIG4) are candidates for breast cancer susceptibility\textsuperscript{28}. Altered repair of DSBs is expected to result in chromosomal instability and rearrangements including translocations, deletions, duplications and inversions, that are characteristic of immortalized and malignant cells. In support of this premise, altered function of proteins involved in sensing DNA damage, DSB repair, and HR promotes or stabilizes rearrangements events that can lead to malignancy. Common polymorphisms in DNA repair genes may alter protein function and capacity to repair damaged DNA; deficits in repair capacity may lead to genetic instability and
carcinogenesis. Therefore, inheritance of repair genes with reduced DNA repair activity is predicted to lead to an increased cancer risk.

XRCC3 is a highly suspected candidate gene for cancer susceptibility. Interestingly, however, contrary to the expected role of variants resulting from polymorphisms of DNA repair genes, a variant of XRCC3, Thr241Met is functionally active for homology-directed repair (HDR), complementing the HDR defects of an XRCC3 mutant cell line as well as the wild-type XRCC3 protein. Extensive case-control studies of this XRCC3 Thr241Met polymorphism in Caucasian, Korean, Portuguese and Thai populations shows its modifying role in the susceptibility to breast cancer. These results suggest that the increased cancer risk associated with this variant may not be due to an intrinsic HDR defect. This further raises the possibility of existence of other biological functions of XRCC3, than DNA repair.

1.3 XRCC3, apoptosis, and cell cycle:

Homologous recombinational repair (HR) is the major pathway involved in the repair of double-strand breaks that are induced by cisplatin, a potent anti-tumor agent, displaying its cytotoxicity by induction of DNA interstrand cross-links (ICLs) that are sensed by RAD51 which guides both recognition and repair by homologous recombination through assembly of multi-enzymatic complexes (RAD51 nuclear foci) containing RAD51 paralog family members. RAD51 paralog-defective hamster cell lines like 1rs1SF, are known to show spontaneous chromosomal aberrations, high sensitivity to killing by cross-linking agents, and attenuated Rad51 focus formation on exposure to ionizing radiation (IR). Where XRCC3 deficiency by HR mediated gene-inactivation in a human
colon cancer cell line HCT116 results in a minor sensitivity to DNA cross-linking agents and increased endoreduplication\textsuperscript{33}, XRCC3 overexpression in breast cancer cell line MCF-7 results in resistance to cisplatin, increased RAD51 foci formation, increased cisplatin-induced S-phase arrest (DNA synthesis arrest) and a decreased cisplatin-induced apoptosis\textsuperscript{34}. These studies emphasize on XRCC3 as a useful marker of prognosis in the efficacy of cross-linking agents in the treatment of human tumors. XRCC3 mediates this cisplatin resistance by RAD51–dependent homologous recombination highlighting the intertwining crosstalk between HR and S-phase checkpoint machinery. The exact molecular mechanism of how XRCC3 is involved in the prolonged S-phase checkpoint is still debated. It is possible that that the prolonged S-phase checkpoint observed in XRCC3-overexpressing cells is the reflection of increased cisplatin-induced DNA repair. It is also possible that the effect on S-phase checkpoint after cisplatin treatment may be due to differences in the stress response to DNA damage in XRCC3-overexpressing cells. These results support the idea that XRCC3 may have functions other than direct involvement in DNA repair. However, since overexpression studies were used, in the presence of DNA damage inducing agents, it is necessary to do direct functional studies examining the effect of XRCC3 loss on relevant endpoints such as growth and apoptosis, as well as other cancer-relevant functions.
2. Hypothesis:
Based on the lowered expression of XRCC3 in progression to invasive phenotype, we hypothesized that it may be a negative regulator of invasion. We aimed to determine the effects of up or downregulation of XRCC3 in invasion using a series of cell lines modeling breast cancer progression. The mechanisms by which this occurs was interrogated by examining the relationship between XRCC3 and other RAD51 paralogs, as well as secreted factors and integrin-mediated cell adhesion.
3. **Materials and Methods:**

3.1: **To determine the effect of siRNA downregulation of XRCC3 on acquisition and maintenance of invasiveness:**

Downregulation of XRCC3 was achieved by using siRNAs for interfering with the XRCC3 gene. Initially, three siRNAs against human XRCC3 gene were used (Ambion–Applied Biosystems; Catalog no. AM16706A; RefSeq no. NM_005432): siRNA-XRCC3-1 (siRNA ID-139504) with sequence (5’-3’)-GGUGGAGAAAGCAUAUCCCtt targeting exon-10; siRNA-XRCC3-2 (siRNA ID-16166) with sequence (5’-3’)-GGUUUUACACUUUUCUGGAtt, targeting exon-5; and siRNA-XRCC3-3 (siRNA ID-16248) with sequence (5’-3’)-GCCUUUGACAAUAAAUGtt targeting exon 10 from 2553 bp – 2571 bp. Out of the three siRNAs, siRNA-XRCC3-3 (ID-16248) worked effectively in downregulating XRCC3 levels as a result of decreased gene transcription, as determined by RT-PCR. Here onwards, this siRNA will be denoted as XRCC3 siRNA-2.

In addition, a siGENOME SMARTpool siRNA (Dharmacon-ThermoScientific; Catalog no. M-012067-01-0005; RefSeq no. NM_001100118) was also chosen to downregulate XRCC3 protein levels. This SMARTpool siRNA consists of a set of four targeting siRNA sequences- siRNA-D-012067-01 with sequence (5’-3’)-GGACCUGAAUCCCGAGAAC targeting exon 4 from 380 bp – 398 bp; siRNA-D-012067-02 with sequence (5’-3’)-GCUGAGAAACCGCCUCCUUA- targeting exon 5 from 509 bp – 527 bp; siRNA D-012067-03 with sequence (5’-3’)-GGACCAGACUUGAAGAGAC- targeting exon 5 from 456 bp – 474 bp; and siRNA D-012067-04 with sequence (5’-3’)-UGAAAUCCGGUAAGGAGGU- targeting exon 5 from 424 bp – 442 bp. Here onwards, this siRNA will be denoted as XRCC3 siRNA-1. siRNA transfection efficiency was
observed by using a Cy3 labeled negative silencer siRNA (negative control) which consists of a scrambled siRNA sequence not intended to target any known gene sequence and tagged to a fluorescent label at its 5` end. Here onwards, this siRNA will be denoted as Scr. siRNA.

**Western blot analysis:** To assess XRCC3 protein levels downregulated using siRNA with respect to the endogenous XRCC3 protein levels in T4-2 cells, a western blot analysis was performed. Cells were trypsinized and the obtained pellets were subjected to treatment with 100 µl of cell lysis buffer (1X RIPA buffer: 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na₂EDTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM Na₃VO₄) followed by sonication to obtain lysates. Protein estimation of lysates was done using BIORAD DC protein estimation kit with serially diluted BSA protein as standards. Following protein quantification, lysates were normalized and run on SDS-PAGE gels (10% or 12% acrylamide gels) with BIORAD Kaleidoscope Precision Plus protein ladder. Gels were transferred overnight onto a nitrocellulose membrane at 20mA, blocked for 60 minutes with 5% milk in TBS-T (Tris Buffered Saline with 0.05% Tween-20 and 0.5g BSA) and probed overnight for XRCC3 protein using anti-XRCC3 antibody- Genetex mouse-monoconal anti-XRCC3 (Catalog no. GTX40254, Clone 10F1/6, 100µg, 1mg/ml) at 1:250 dilution or Novus Biologicals mouse monoclonal anti-XRCC3 (Catalog no. NB100-180, Clone 10F1/6, 1.0 mg/ml) at 1:1000 dilution. Membranes were treated with anti-mouse LICOR Secondary antibody (1:5000 dil) for 2 hrs at room temperature and viewed under LICOR Imager (Odyssey v1.2 software) at a wavelength of 800 nm. Membranes were reblocked and probed for
β-actin or α-Tubulin protein (loading control). β-actin or α-Tubulin, being ubiquitous cytoskeletal proteins, were used as a loading control to deduce the relative amount of protein loaded for each sample. The bands were quantified using Odyssey LICOR infrared imaging software v1.2.

**RT-PCR analysis:** To assess the siRNA mediated inhibition of XRCC3 at the mRNA level in T4-2 cells, the levels of XRCC3 transcripts were monitored by semiquantitative RT-PCR analysis. Using TRIzol (Guanidinium thiocyanate-phenol-chloroform) extraction kit (Invitrogen), RNA is prepared from the cell pellets by lysis with trizol reagent, phase separation with chloroform, RNA precipitation with isopropyl alcohol, RNA washing with 75% ethanol and redissolving the RNA in nuclease-free-water. RNA concentrations were deduced using a spectrophotometer (Eppendorf Biophotometer Plus). By convention, the absorbance at 260 nm of 40µg/ml solution of single stranded RNA is assumed to be equal to 1. The OD$_{260}$/OD$_{280}$ value gives us the purity of RNA, a value of 2.0 being highly pure and a value of 1.6 or lower being not acceptable. The concentration of RNA was recorded in µg/ml. cDNA was made from RNA using Thermoscript RT-PCR system (Invitrogen) where single stranded RNA is converted to single stranded DNA using reverse-transcriptase enzyme. The PCR reaction was carried out using Eppendorf authorized thermocycler within a suitable range of 25 - 35 cycles (linear range determined empricially) with primers for XRCC3 and GAPDH (internal control). Primers used for XRCC3: forward (5’-3’) - tcctgttctacagcatgc and reverse-gacactggagttgggtgatat. Primers for GAPDH: forward (5’-3’) - cccctggcaggtcatccag; and GAPDH reverse- cataccagaaagtgttgcaaaag. The
PCR products were loaded onto 1.5% agarose gels and run at 150 volts for 75 minutes with 1 kb full scale standard DNA ladder (Fischer Scientific). Band for XRCC3 observed at 643 bp and for GAPDH at 490 - 500 bp respectively were quantified using Adobe Acrobat photoshop. The siRNA inhibition experiment and RT-PCR was repeated four times starting with freshly thawed T4-2 cells.

**Invasion assay:** To evaluate the effect of XRCC3 downregulation on the invasive potential of T4-2 cells, invasion assays were performed. In a 24-well plate, 1x10^5 cells were plated (in triplicates) into cell well inserts on a thin layer of Laminin-rich ECM (Matrigel™- which mimics the basement membrane) over a 8.0 µm holed mesh. After an incubation of 48 hours at 37°C in the presence of 5% CO₂ using H14 medium in both the upper chamber into which cells were plated and the lower chamber towards which they will invade (regular growth conditions for T4-2 cells; no chemotactic gradient); the number of cells that invade through the matrix and mesh were fixed with gluteraldehyde, stained with toluidine blue stain and counted. Cell counting of each cell well insert or well was done field by field (ranging from 12-15 fields under 200X magnification/well) and each well was counted at least twice. The total number of invading cells in each well were counted and divided by the no. of 200X fields used for counting. The mean value (from cell counts of triplicate wells) of each sample was calculated. Graph was plotted with mean values of scrambled control siRNA vs XRCC3 siRNA on the x-axis and Avg. no. of invading cells / 200X field of microscope on the y-axis. Standard deviation (from triplicate wells) of each sample was calculated and represented as y-error bars. Experiment was repeated four times. The mean values of scrambled control
siRNA from each experiment were normalized to 1 and the respective XRCC3 siRNA mean values were divided by the scrambled control siRNA mean values to obtain normalized averages. A combined graph \((n=4)\) was plotted with normalized average values of scrambled control siRNA vs. XRCC3 siRNA on the \(x\)-axis. Standard deviation of normalized average values of each sample (from 4 experiments) was calculated and represented as \(y\)-error bars documenting variability across experiments. Student’s t-test (2-tailed; type-2) was performed using normalized average values of scrambled control siRNA (from 4 experiments) vs the respective normalized average values of XRCC3 siRNA treated cells to obtain value of significance i.e. \(P \leq 0.05\).

**Cell proliferation assay:** To determine the number of viable / metabolically active cells, a calorimetric estimation of scrambled control siRNA transfected and XRCC3 siRNA transfected T4-2 cells was done using the Cell Titer 96 Aqueous One solution Cell Proliferation Assay™ (Promega). This assay is based on a novel MTS Tetrazolium compound (Owen’s reagent). This MTS compound is bioreduced by active cells into a colored Formazan product. This is accomplished by NADPH / NADH produced by dehydrogenase enzymes in active cells. A standard curve was produced using a varying number of cells plated at time \(= 0\) and determining how many cells are needed in order to stay in the linear portion of the curve. This was done at 2, 4, 24 and 48 hrs after plating and it was determined that plating 1000, 5000 or 10000 cells at time \(= 0\) produced absorbance values within the linear part of the curve. Fixed populations of 1000, 5000, 10000 cells, each of scrambled control siRNA and 2 XRCC3 siRNA transfected T4-2 cells were checked for absorbance at 490 nm with a 96-well
spectrophotometric plate reader after 6, 12, 24, 36 and 48 hours respectively. Three experiments were performed, each with 3 cell populations of 1000, 5000 and 10000 cells of scrambled control siRNA, XRCC3 siRNA-1 and XRCC3 siRNA-2 transfected T4-2 cells (in triplicates) at 6, 12, 24, 36 and 48 hours. In each experiment, the mean value (spectrophotometric readings; triplicate wells) of scrambled control siRNA at 6 hours timepoint was normalized to 1, and all other mean values were divided by the scrambled control siRNA mean value at 6 hours to obtain normalized averages. For each siRNA, at every time-point, a mean value (from normalized average values; 3 experiments) was calculated. A combined graph (each for 1000, 5000, 10000 cells) was plotted with these mean values of scrambled control siRNA vs XRCC3 siRNA-1 and XRCC3 siRNA-2 at 6, 12, 24, 36 and 48 hours. Standard deviation from those mean values of each sample was calculated and represented as y-error bars to document variability across experiments. Student’s t-test (2-tailed; type-2) was performed using the same mean values of scrambled control siRNA vs. the respective mean values of XRCC3 siRNAs to obtain value of significance i.e. $P \leq 0.05$.

**TUNEL apoptosis assay:** To determine the effect of siRNA associated apoptosis, In situ Cell death Detection Kit, Fluorescein™ (Roche) was used according to the manufacturer’s protocol for adherent cells. 35000 cells were needed for the standard cell density of T4-2 cells on tissue culture plastic when seeded onto 4-well chambered glass slides. 35000 cells each of scrambled control siRNA, XRCC3 siRNA-1, XRCC3 siRNA-2 transfected T4-2 cells were plated (in duplicates) in the 4-well chamber slides plus two wells of untreated T4-2 cells (for negative and positive controls of assay) for
12, 24, 36 and 48 hrs each. At each time point, cells were fixed using 4% paraformaldehyde in PBS, then permeabilised using 0.1% Triton X-100 in 0.1% sodium citrate at 4°C (on ice) for 2 minutes and then labeled with TUNEL reaction mixture (1 hour incubation at 37°C in a humidified chamber). DNase1 treated T4-2 cells were used as a positive control. The TUNEL reaction preferentially labels DNA strand breaks generated during apoptosis. Labeling of DNA strand breaks by Terminal deoxynucleotidyl transferase catalyses polymerization of labeled nucleotides to free 3’OH DNA ends in a template – independent manner. Fluorescein labels, incorporated in nucleotide polymers, were detected and quantified under the fluorescence microscope after 12, 24, 36 and 48 hrs by using an excitation wavelength of 475 nm and detection in the range of 515 – 565 nm. DAPI staining was used to determine the total number of nuclei and the % number of apoptotic cells was calculated as (Fluorescein$^{\text{positive}}$ / DAPI). At least 8 randomly chosen 10X fields of the microscope were counted per well of the 4-well chambered slide for each sample. At each time-point, the mean values (from duplicate wells; Avg.no. of apoptotic cells-fluorescein$^{\text{positive}}$/ DAPI) for each sample were calculated. Graph was plotted with mean values of scrambled control siRNA vs. XRCC3 siRNA-1 and XRCC3 siRNA-2 at 12, 24, 36 and 48 hour timepoints on the x-axis and avg. no. of apoptotic cells on the y-axis. Standard deviation from these mean values (duplicate wells) of each sample was calculated and represented as y-error bars. Experiment was repeated three times. In each experiment, the mean values of scrambled control siRNA at 12 hours timepoint were normalized to 1 and all other mean values were divided by the scrambled control siRNA mean value at 12 hours to obtain normalized averages. For each siRNA, at every
time-point, a mean value (from normalized average values; 3 experiments) was calculated. A combined graph was plotted using these mean values of scrambled control siRNA vs. XRCC3 siRNA-1 and XRCC3 siRNA-2 at 12, 24, 36 and 48 hours. Standard deviation from these mean values of each sample was calculated and represented as y-error bars, documenting variability across experiments. Student’s t-test (2-tailed; type-2) was performed using the same mean values of scrambled control siRNA vs. the respective mean values of XRCC3 siRNAs to obtain value of significance i.e. $P \leq 0.05$.

3.2. To determine the role of RAD51 paralogs found in complex with XRCC3 in Invasion

A. Does XRCC3 siRNA change the levels of any of the paralogs?

Western Blotting: Following siRNA mediated downregulation of XRCC3, whole cell lysates were prepared and run on SDS-PAGE gels. Lysates were probed for XRCC3 protein to confirm downregulation of XRCC3 levels in both XRCC3 siRNA-transfected T4-2 cell lysates as compared to scrambled control siRNA. The same lysates were probed for RAD51B, RAD51C and RAD51D proteins using specific primary antibodies-

Mouse monoclonal anti-RAD51B- Novus Biologicals (Catalog no.NB100-176, clone 1 H3/13, at 1:1000 dilution); Mouse monoclonal anti-RAD51C–Novus Biologicals (Catalog no. NB100-177, Clone 2H11/6 at 1:1000 dilution); Mouse monoclonal anti-RAD51D–Novus Biologicals (Catalog no.NB100-178, clone 5B3/6 at 1:1000 dilution). Experiment was repeated twice. Quantitative band densitometric analysis was performed on the bands to calculate fold reduction as compared to loading controls- α-tubulin and β-actin.
B. Do other RAD51 paralogs function in invasion with XRCC3?

**siRNA mediated downregulation of RAD51C:**

Downregulation of RAD51C was achieved by using siGENOME smartpool siRNAs (Dharmacon-Thermoscientific; Catalog no. M-010534-01-0005; Human RAD51C; RefSeq no. NM_002876). This SMARTpool siRNA consists of a set of four targeting siRNAs sequences- siRNA D-010534-02 with sequence (5´-3´)-GUACAGCACUGGAACUUCU targeting exon 2 from 757 bp – 775 bp; siRNA D-010534-03 with sequence (5´-3´)-GCAGAAGCCUUAGAAACUC targeting exon 2 from 666bp-684bp; siRNA D-010534-04 with sequence (5´-3´)-GGAGUGCCCUUAAUGAAAA targeting exon 2 from 843 bp – 861 bp; and siRNA-010534-17 with sequence (5´-3´)-GGUGAAAACCCUCGCAGCUU targeting exon 1 from 623 bp – 641 bp.

**Western blotting:** Following siRNA mediated downregulation of RAD51C, whole cell lysates were prepared at 24 hours, 36 hours, 48 hours and run on SDS-PAGE gels to check for RAD51C protein downregulation. α-tubulin was used as loading control.

**Invasion assay:** To evaluate the effect of RAD51C downregulation on the invasive potential of T4-2 cells, invasion assay was performed with RAD51C siRNA vs scrambled control siRNA transfected T4-2 cells (in triplicates).
3.3. **Determine the effect of XRCC3 siRNAs on canonical invasion pathways**

**A. Test the effect of XRCC3 siRNA on the ability of T4-2 conditioned medium to induce invasiveness in T4-2 cells (secreted factors):**

**Invasion assay:** T4-2 cells were transfected with scrambled control siRNA and XRCC3 siRNAs for 48 hours. To determine the effect of altered XRCC3 on factors secreted from T4-2 cells that are important for the invasiveness of T4-2 cells, a set of invasion assays was performed with untreated T4-2 cells in growth medium alone, T4-2 cells in medium with conditioned medium (CM) from T4-2 cells treated with XRCC3 siRNA-1, CM from T4-2 cells treated with XRCC3 siRNA-2 or from T4-2 cells in medium with CM from T4-2 cells transfected with scrambled control siRNA. CM was mixed with DMEM-F12 at 25%, 50%, or 75% by volume and additives were added to the final volume.

**B. Test the effect of XRCC3 siRNA on cell surface integrins and activation of FAK:**

**Western blotting:** Following siRNA mediated inhibition of XRCC3 in T4-2 cells, cell lysates were prepared as mentioned above and membranes were probed for total FAK, FAK phosho-Y397 and FAK -Y861 using specific antibodies. Mouse monoclonal anti-FAK (BD Transduction laboratories, Clone- 77/FAK, Catalog no.610088, 150µg, 250µg/ml, 1:500 dilution); Mouse monoclonal anti-FAK-pY397 (BD Transduction laboratories, Catalog no. 611722, 50µg, 250µg/ml at 1:250 dilution.)
**Indirect immunofluorescence:** To study the FAK and phospho-FAK containing adhesion complexes in XRCC3 siRNA and control treated T4-2 cells, immunostaining was performed. 35000 cells (XRCC3 siRNAs vs. scrambled control siRNA transfected T4-2 cells) were plated onto each well of a four-well chambered slide and incubated for 24-48 hrs. After incubation, cells were washed with PBS (pH 7.4), permeabilized by Triton extraction and fixed by 4% paraformaldehyde treatment. Cells were treated with freshly prepared 0.5% Triton-100 in CSK buffer (10mM Pipes, pH 6.8, 10mM NaCl, 300mM sucrose, 3mM MgCl₂ and 2mM EDTA) with protease inhibitor (1:100 dilution) for 10 minutes on ice and then washed twice with just CSK buffer with protease inhibitor for 5 minutes each. Cells were further treated with freshly prepared 4% paraformaldehyde in CSK buffer (pH 7.4) at room temperature for 20 minutes and washed thrice with PBS / Glycine for 20 minutes each. The slides were further blocked using 10% goat serum in IF buffer for 1 hour at room temperature. Primary antibodies were added to 10% goat serum in IF buffer (1:50-1:100 dilution / 300µl volume / well), and the chambered slides were wrapped with parafilm, or covered using coverslips, placed in a humidified chamber and stored overnight at 4°C with gentle agitation. The next day, slides were washed thrice with IF buffer for 20 minutes with gentle agitation and fluorescent anti-mouse or anti-rabbit secondary antibody (Alexa Fluor-488) in 10% goat serum, in IF buffer (1:400 dilution / 300µl volume / well) was added for 1 hour, protected from light. Slides were further washed thrice with PBS / Glycine for 20 minutes each followed by nuclear staining using DAPI (1:10000 dilution / 300µl volume / well) for 5-10 minutes at room temperature. Slides were finally washed once with IF buffer, chambers were removed, Vectashield was added, coverslips were placed and slides were viewed under
100X field of confocal microscope. One well of one of the 4-well chambered slides contained untreated T4-2 cells with no primary antibody acting as a negative control to determine any non-specific binding by the secondary antibody. For positive controls, T4-2 cells transfected with the various siRNAs were treated with growth factors like EGF (20µg/ml) and TGFβ (2ng/ml) at the time of seeding on 4-well chambered slides. Treatment with EGF alone was used as positive control for total FAK and FAK-Y861 antibodies and treatment with EGF+TGFβ was used as positive control for FAK-Y397 antibody. Confocal microscopy was employed (using LSM-510 laser scanning microscope) to compare the signal from XRCC3 siRNA transfected T4-2 cells to the scrambled control siRNA transfected cells, leaving exposure and acquisition settings constant for the compared images.

**Flow Cytometry:** To study the cell surface integrin expression, FACS analysis was performed on si-XRCC3 treated vs. scrambled control siRNA transfected T4-2 cells. Cells were transfected with siRNAs for 48 hrs and trypsinized as usual. Aliquots of 500000 cells of each kind (in triplicates), were collected in 1.5ml microcentrifuge tubes and spinned down at 5000 rpm. Media was removed. Cells were washed with 1000µl of ice-cold FACS buffer (1X PBS, 5% Fetal Bovine Serum and 0.1% sodium azide). Cells were centrifuged and washed thrice with FACS buffer (on ice). Primary antibodies:

- Total beta-1 integrin (Mouse monoclonal Anti-Human Integrin Beta1 (Chemicon international, Catalog no.MAB1959, Clone-P5D2, 100µg, 1mg/ml) and Mouse monoclonal Activated Beta-1 integrin CDC29 were added at 1:10 dilution in 50µl of FACS buffer, separately and cells were incubated in it for 1 hour at 4°C with repeated
pipetting, after every 15 minutes, to keep cells suspended during incubation. Cells were washed thrice with FACS buffer. Anti-mouse secondary antibody (Alexa Fluor-488) at 1:100 dilution in 50µl of FACS buffer was added to the tubes and cells were incubated for 30 minutes on ice, with repeated slow pipetting every 15 minutes to ensure cells were suspended. Cells were washed thrice with FACS buffer. After the last wash, cells were resuspended in 100 µl of freshly prepared 2% paraformaldehyde in PBS+ 0.1% sodium azide. Untreated T4-2 cells and T4-2 cells treated with just secondary antibody were used as controls.
4. Results:

4.1. Model used: Human breast cell line model of pre-invasive to invasive transition\textsuperscript{5}.

A series of breast epithelial cell derived cell lines called the HMT-3522 series were used as a model of breast cancer progression to invasiveness. The HMT-3522 progression series has both pre-invasive (S3) and invasive (T4-2) counterparts, resembling the basal-like subtype of breast cancers at the molecular level by gene expression profiling. HMT-3522 human breast epithelial cell line series (S1, S2, S3 and T4-2) were derived from a reduction mammoplasty specimen of a patient with fibrocystic breast disease. The epithelial component of the tissue was grown in defined medium to give rise to S1 cells that became immortalized spontaneously, are EGF-dependent for growth, and are non-tumorigenic. S2 cells were derived from S1 by continuous culturing in the absence of EGF. T4-2 cells are tumorigenic, and were derived from S2 cells by inoculating mice, which eventually produced a rare tumor, which was cultured and passaged again through mice. S3 cells are non-invasive but have a higher potential for acquiring invasiveness than the parent S2 and S1 when treated with conditioned medium (CM) from T4-2 cell cultures. These cell series were grown on a three-dimensional laminin-rich basement membrane (3DlrBM) to assay both the normal breast and breast cancers. In 3DlrBM, S1 cells produce growth-arrested acini that express differentiation markers mimicking \textit{in vivo} polar patterns of localization of a number of markers. S2 colonies are heterogeneous and T4-2 cells form large and disorganized colonies. S3-A, S3-B, and S3-C are noninvasive but had a higher potential for acquiring invasiveness than the parent S2 cells.
There are substantial similarities between the model of S3 vs. T4-2 and pre-invasive to invasive transition in breast carcinogenesis. S3s are less tumorigenic than T4-2 in immunocompromised nude mouse xenografts, producing ER / PR / HER2 triple negative, i.e. tested negative for estrogen receptors (ER-), progesterone receptors (PR-), human epidermal growth factor receptor 2 (HER2-) and CK5/6 HER1 positive tumors with similarity to gene expression profiles reported for the basal subtype of breast cancers.

![Figure 4.1: HMT3522 series of cell lines.](image)

### 4.2. Rationale for testing XRCC3: XRCC3 expression alterations in progression:
Previous unpublished data from Dr. Aylin Rizki shows that XRCC3 is also found downregulated in invasive T4-2 cells as compared to pre-invasive S3 cells grown in 3DlrBM (Figure 4.2). Since XRCC3 levels dropped as cells became more invasive, these results prompted the question of whether XRCC3 might play a negative role in the acquisition and maintenance of invasiveness in breast epithelial cancer cell progression.
Figure 4.2: Western blot for XRCC3 protein: S1, S3-A, S3-B, S3-C and T4-2 cells were grown in 3DlrBM for 10 days. Top panel, XRCC3 western blot; Bottom panel, ponceau stain used as a loading control to show even loading across the cell lines around the molecular weight of XRCC3.
4.3. **Rationale for testing XRCC3: XRCC3 overexpression effects on invasion and growth:**

Previous unpublished data shows that XRCC3 overexpression in invasive T4-2 cells using LXSN retroviral transduction (Figure 4.3) leads to a decrease in cell number (Figure 4.4) and a 3-fold decrease in invasiveness in invasion assays (Figure 4.5) compared to control cells. These data are consistent with the role of XRCC3 as a negative regulator of invasion. However, since the number of cells in the XRCC3 overexpressing cells were lower than control cells during the time course of the experiment, the decreased number of invading cells could also be the result of there being fewer viable cells in the XRCC3 overexpressing cell samples.

Therefore, we decided that long-term overexpression of XRCC3 would not allow us to determine if it has a direct role in invasion and decided to alter XRCC3 levels for only a short period of time and test if this would alter invasion without altering cell numbers.
Figure 4.3: Western Blot showing XRCC3 protein levels: T4-2 cells were transduced with retroviral LXSN and LXSN-XRCC3 vectors and lysates were prepared. T4-LXSN and T4-LXSN-XRCC3 lysates were probed for XRCC3 protein. The non-specific band serves as loading control.
**Figure 4.4: Phase contrast images of transduced T4-2 cells:** Phase contrast microscopy images showing LXSN retrovirus transduced T4-2 cells (control) as compared to T4-LXSN-XRCC3 transduced cells after long-term selection, 4 day culture.
Figure 4.5: Effect of XRCC3 overexpression on invasion: Boyden chamber invasion assay results of T4-LXSN control cells vs. T4-LXSN-XRCC3 transduced cells, overexpressing XRCC3: A. Three invasion assay images each of T4-LXSN (top) and T4-LXSN-XRCC3 (bottom) transduced T4-2 cells; B. Figure shows a graph representing mean values of invasion assay counts of T4-LXSN vs. T4-LXSN-XRCC3 with average no. of invading cells per 200X field of microscope on the y-axis. 3 experiments, t-test, $P=0.0001$, relative to T4-LXSN control.
4.4. Effect of siRNA downregulation of XRCC3 on acquisition and maintenance of invasiveness:

Previously, we showed that stable long-term overexpression of XRCC3 in T4-2 downregulated invasion. Since this was accompanied by a decrease in cell number, we set out to determine whether siRNA downregulation of XRCC3 would provide a more direct link to invasion. In order to verify the role of XRCC3 in invasiveness, we tested the effect of downregulating XRCC3 for a short period of time using siRNAs, on the invasiveness of T4-2 cells. This approach also had the advantage that in the short time course of the experiment and the few cell divisions which occurred, it was not possible to induce genome instability, followed by selection for invasive cells. Therefore, the effects of XRCC3 observed on invasion were more likely to be due to a direct role, and not via genome instability.

To test this hypothesis, we transfected T4-2 cells with two independent sets of XRCC3 siRNAs targeting non-overlapping sequences of the gene. The effect of XRCC3 siRNA-1 and XRCC3 siRNA-2 on the mRNA and protein levels were determined by RT-PCR and western blot analysis, respectively (Figure 4.6).

Quantitative band densitometry analysis of RT-PCR bands showed a 6-fold reduction in XRCC3 levels as compared to scrambled control siRNA. Western blotting results show 50-60% reduction of XRCC3 protein in XRCC3 siRNA transfected T4-2 cells vs. untreated T4-2 and Cy3 labeled scrambled control siRNA transfected T4-2 cells. The downregulation of XRCC3 occurred by 48 hours post-transfection and was stable for 2-3 days.
**Figure 4.6: siRNA downregulation of XRCC3:** (page 31) **A.** Fluorescence Microscopy images of Cy3 labeled- scrambled control siRNA transfected T4-2 cells, two days post-transfection. Blue arrows, localization of florescent Cy3 labels, inside cells (shown in the image below with phase contrast); **B.** RT-PCR analysis for monitoring XRCC3 mRNA levels in T4-2 cells transfected with scrambled control siRNA vs. XRCC3 siRNA-2 with GAPDH as loading control. PCR (10µL rxn) was run with 35 cycles at annealing temperature of 65°C using 3 cDNA dilutions– 1:10, 1:50 and 1:100, each of scrambled siRNA vs. XRCC3 siRNA. The PCR products for XRCC3 and GAPDH were observed at 643bp and 500bp respectively; **C.** Western blot analysis showing downregulation of XRCC3 protein using 2 siRNAs against XRCC3 vs. untreated T4-2 cells and scrambled control siRNA transfected T4 cells. α-Tubulin was used as internal loading control. Bands for XRCC3 and α-Tubulin were observed at 38kD and 50kD, respectively.
4.5. **Effect of XRCC3 siRNA on the Boyden chamber / transwell Invasion assay based invasiveness of T4-2 cells:**

In order to test the effect of XRCC3 downregulation on invasiveness through ECM, we first downregulated XRCC3 using siRNAs for 48 hrs and used these cells and control siRNA treated T4-2s to determine the effect on invasion. In a 24-well plate, 1x10^5 cells each of XRCC3 siRNA-1, XRCC3 siRNA-2 and scrambled control siRNA transfected T4-2 cells were plated into a cell well insert on a thin layer of Laminin-rich ECM (Matrigel™) with an 8.0 µm mesh. After 48 hours, the number of cells that invaded through the matrix and mesh were fixed, stained with Toluidine blue stain and counted under 200X field of microscope.

Results from the invasion assays show a significant 3-fold increase in invasiveness in XRCC3 siRNA transfected T4-2 cells as compared to scrambled control siRNA (Figure 4.7), highlighting the role of XRCC3 as a negative regulator of invasion.
Figure 4.7: Effect of transient downregulation of XRCC3 on invasiveness of T4-2 cells through laminin-rich basement membrane (lrBM) in Boyden chamber assays or transwell invasion assays:  

A. 200X field image of invading cells, each from cell well inserts of scrambled control siRNA vs. XRCC3 siRNA-2 transfected T4-2 cells;  

B. Invasion assay results of scrambled siRNA vs. XRCC3 siRNA-2 transfected T4-2 cells from 4 sets of experiments (n=4). Mean values (from triplicate wells; Avg. no. of invading cells / 200X field / well) of scrambled siRNA from each experiment were normalized to 1 and the XRCC3 siRNA-2 mean values were divided by scrambled siRNA mean values to obtain normalized averages. x-axis of the graph was plotted with normalized average values of scrambled siRNA vs. XRCC3 siRNA. Standard deviation across these values for each sample is plotted as y-error bars. y-axis represents fold difference with respect to scrambled siRNA values, normalized to 1. Four experiments, t-test, \( P = 0.0004 \), compared to scrambled control siRNA.
4.6. **Cell proliferation assay for the effect of XRCC3 siRNA on T4-2 cells:**

In order to determine whether XRCC3 siRNAs affect cell proliferation during the time course of the invasion assay, we performed MTT assays. The goal was to determine if XRCC3-si could result in higher invading cell numbers simply by increasing proliferation and therefore the number of cells available to invade.

To determine this, a fixed number of T4-2 cell populations (1000, 5000, or 10000 cells) were seeded into 96-well plates, in triplicates. MTT-based cell viability assay was performed and the wells were analyzed by spectrophotometer at the wavelength of 490nm after 2, 6, 24 and 48 hours. This allowed us to determine the number of cells to be used for the MTT assay during the time-course of the invasion assay. 1000, 5000 and 10000 cells produced results within the linear part of the curve, especially beginning with the 6 hr time point.

We then used T4-2 cells after treatment with control and XRCC3 siRNAs for 48 hrs to ensure XRCC3 downregulation and determined the effect of this on proliferation using the MTT assay at 6, 12, 24, 36, and 48 hrs after plating the cells. Results from the MTT assay show no significant change in the growth and viability of both XRCC3 siRNA transfected T4-2 cells vs. scrambled control siRNA (Figure 4.9). These results suggest no effect of XRCC3 siRNAs on the growth of T4-2 cells as compared to control siRNA, during the 48 hours time course of the invasion assay. Therefore, the observed increase in invasion by XRCC3 downregulation could not be due to an unintended increase in proliferation.
Figure 4.8: Optimizing MTT for T4-2 cells: Fixed populations (1000, 5000, 10000 cells) of T4-2 cells were seeded into 96-well plates. Wells were analysed by spectrophotometer at the wavelength of 490nm after 2, 6, 24 and 48 hours respectively. The graph represents mean values of spectrophotometric readings for each cell population (triplicate samples) at 2, 6, 24, 48 hours with error bars at each time-point derived from their respective standard deviation values.
Figure 4.9: Effect of XRCC3 siRNA on T4-2 cell proliferation / growth: (page 37)

Three MTT experiments were performed, each with 1000, 5000 and 10000 cells of scrambled control siRNA (black), XRCC3 siRNA-1 (red) and XRCC3 siRNA-2 (green) transfected T4-2 cells (in triplicates) at 6, 12, 24, 36 and 48 hours. Normalized average values were obtained, as described in Methods. At every time-point, for each siRNA, a mean value* (from normalized average values; 3 experiments) was calculated. In the figure, the graphs for 1000, 5000 and 10000 cells were plotted with these mean values of scrambled control siRNA, XRCC3 siRNA-1, XRCC3 siRNA-2 at 6, 12, 24, 36 and 48 hours. Standard deviation from these mean values for each sample was calculated and represented as y-error bars. 3 experiments, t-test, $P > 0.05$, compared to scrambled control siRNA values (6-48 hrs).
4.7. **TUNEL apoptosis assay for the effect of XRCC3 siRNA on T4-2 cells:**

In order to determine if XRCC3 downregulation effect on increasing invasion could be due to a lower number of apoptotic cells being present, we did TUNEL assays determining the effect of XRCC3 siRNAs vs. scrambled siRNA on apoptosis during the time course of the invasion assay.

To estimate the amount of apoptosis associated with normal T4-2 cells and optimize conditions, fixed number (35000 T4-2 cells/well) were seeded into the wells of 4-well chambered slides as described above in Methods. After 24 hours, the cells were fixed, permeabilized, treated with enzyme and labeling solution and fluorescein labels incorporated in nucleotide polymers were detected and quantified under the fluorescence microscope by using an excitation wavelength of 475 nm and detection in the range of 515 – 565 nm. DAPI staining was used to determine the total number of nuclei. DNase 1 treated T4-2 cells act as positive control for the assay (Figure 4.10 A. right panel), and cells treated just with labeling solution, but no enzyme act as negative control (Figure 4.10 A. left panel) Results showed approximately 9-10% apoptotic T4-2 cells (Figure 4.10 A. middle panel, data not shown).

We then compared the apoptosis in XRCC3 siRNAs vs. scrambled control siRNA transfected T4-2 cells (after 48hrs at which downregulation was confirmed by western blot) at 12, 24, 36 and 48 hrs (Figure 4.10 B). Results from the TUNEL apoptosis assay show no significant effect of XRCC3 siRNAs on apoptosis of T4-2 cells as compared to scrambled control siRNA, during the 48 hour time course of the invasion assay. This suggests that the observed increase in invasion could not have been due to a decrease in apoptotic cells in the XRCC3si treated cells.
Figure 4.10: Effect of XRCC3 siRNAs on apoptosis: (page 40) A. TUNEL apoptosis assay images (top- TUNEL; bottom- DAPI) with T4-2 cells treated without enzyme (negative control- labeling solution alone); T4-2 cells treated with labeling solution + enzyme; and T4-2 cells treated with 300U/ml of DNase 1+ labeling solution + enzyme (positive control); B. TUNEL apoptosis assay results from 3 sets of experiments, each of which had 3 samples – scrambled control siRNA (black), XRCC3 siRNA-1 (red), XRCC3 siRNA-2 (green) transfected T4-2 cells, in duplicates, at 12, 24, 36, 48 hrs, respectively, during the 48 hour time course of invasion assay. Average no. of apoptotic (Fluorescein\textsuperscript{positive}/DAPI) cells were counted, mean values and normalized average values were calculated, as described in Methods. For each siRNA, at every time-point, a mean value* (from normalized average values; 3 experiments) was calculated. In the figure, the graph was plotted using these mean values of scrambled control siRNA vs. XRCC3 siRNA-1 and XRCC3 siRNA-2 at 12, 24, 36 and 48 hrs, respectively. Standard deviation from mean values of each sample was calculated and represented as y-error bars. 3 experiments, t-test, $P > 0.05$, compared to scrambled control siRNA (at 48 hr timepoint): $P= 0.225$ (XRCC3 siRNA-1 vs. scrambled control siRNA) and $P= 0.188$ (XRCC3 siRNA-2 vs. scrambled control siRNA).
A. Negative control (labeling solution alone)  Positive control (Dnase 1 treatment + labeling solution + Enzyme)

B. 

*Average no. of Apoptotic (Fluorescein+/DAPI) cells

n=3

- Scr. siRNA
- XRCC3 siRNA-1
- XRCC3 siRNA-2

12hr  24hr  36hr  48hr
4.8. **Effect of XRCC3 siRNAs on RAD51 paralog levels:**

XRCC3 being a DNA repair gene, acts in concert with RAD51 paralogs B, C, D and XRCC2 to repair DNA breaks. To see if these interactions are important in terms of invasion, as they have been demonstrated for DNA repair, we studied the effect of XRCC3 downregulation on the levels of RAD51 paralogs.

For this, we treated T4-2 cells with either scrambled control or the two XRCC3 siRNAs for 48 hours and used the lysates to determine the level of XRCC3, RAD51B, RAD51C, and RAD51D. Results show no significant change in the levels of RAD51B (insignificant \( P \) values for both XRCC3 siRNAs vs. scrambled siRNA) or RAD51D (insignificant \( P \) values for both XRCC3 siRNAs vs. scrambled siRNA), when XRCC3 was downregulated (with significant \( P \) values for both XRCC3 siRNAs vs. scrambled siRNA). Levels of RAD51C protein were lowered (with significant \( P \) values for both XRCC3 siRNAs vs. scrambled siRNA) in XRCC3 downregulated T4-2 cell lysates (Figure 4.11). These results show that in T4-2 cells as well as in HT1080 and HeLa cells previously reported, lowering XRCC3 levels could destabilizes the RAD51C-XRCC3 complex, resulting in a decrease in RAD51C levels.
Figure 4.11: Effect of XRCC3 siRNA on RAD51 paralog levels: (page 43) Left Panel: Sample western blot from a single XRCC3 downregulation experiment. T4-2 cells were transfected with scrambled-control siRNA, XRCC3 siRNA-1 and XRCC3 siRNA-2 and whole cell lysates were prepared, 48 hours post siRNA transfection. The lysates were probed for XRCC3, RAD51B, RAD51C and RAD51D proteins, respectively. β-actin and α-tubulin were used as loading controls. Right Panel: Quantified avg. band densitometric values of the respective proteins (protein / loading control) normalized to scrambled control siRNA from 2 sets of XRCC3 downregulation experiments (n=2). t-test, P values- For XRCC3 protein: P=0.026 (XRCC3 siRNA-1 vs. scr.siRNA) and P=0.034 (XRCC3 siRNA-2 vs. scr. siRNA); For RAD51B protein: P=0.709 (XRCC3 siRNA-1 vs. scr. siRNA) and P=0.499 (XRCC3 siRNA-2 vs. scr. siRNA); For RAD51C protein: P=0.046 (XRCC3 siRNA-1 vs. scr. siRNA) and P=0.032 (XRCC3 siRNA-2 vs. scr. siRNA); For RAD51D protein: P=0.77 (XRCC3 siRNA-1 vs. scr. siRNA) and P=0.599 (XRCC3 siRNA-2 vs. scr. siRNA).
4.9. **Downregulation of RAD51 C and its effect on invasion:**

Since downregulation of XRCC3 also downregulated RAD51C, we wanted to determine if RAD51C could have a similar function in invasion as does XRCC3. RAD51C was downregulated using smartpool siRNA and lysates were prepared from RAD51C siRNA and scrambled control siRNA transfected T4-2 cells after 24, 48 and 72 hours, respectively, to check for downregulation of RAD51C protein (Figure 4.12 A). This showed successful downregulation up to 72 hrs post transfection.

In order to test the effect of RAD51C on T4-2 cell invasiveness, we transfected the control and RAD51C siRNAs and plated the cells for invasion after 48hrs also demonstrating downregulation by western blots in each experiment. Results from the invasion assay performed on RAD51C siRNA transfected T4-2 cells show a 3-fold significant increase in invasiveness as compared to scrambled control siRNA, with a significant $P$ value of 0.0233, compared to scrambled control siRNA (Figure 4.12 C). This shows that RAD51C is not only dependent on XRCC3 for its stability but also has a function in invasion as a negative regulator, as does XRCC3.
Figure 4.12: siRNA mediated downregulation of RAD51C and its effects on invasion: (page 46) A. Western blot analysis with lysates from scrambled control siRNA and RAD51C siRNA transfected T4-2 cells after 24, 48 and 72 hours post transfection. B. Effect of transient downregulation of RAD51C using siRNAs on invasiveness of T4-2 through laminin-rich basement membrane (lrBM) in Boyden chamber assays or transwell invasion assays: Invasion assay images of scrambled control siRNA and RAD51C siRNA transfected T4-2 cells. Cell counts were obtained from triplicate wells of each sample with average number of invading cells per 200x field; C. Graph showing invasion assay results of scrambled control siRNA vs. RAD51C siRNA transfected T4-2 cells (in triplicate wells) with average no. of invading cells per 200X field of the microscope on y-axis. Two experiments, t-test, P= 0.02, compared to scrambled control siRNA.
A.

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<th>48hrs</th>
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<th>72hrs</th>
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<td>Scr.</td>
<td>si-RAD51C</td>
<td>Scr.</td>
<td>si-RAD51C</td>
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<tr>
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<td><img src="image2" alt="Image" /></td>
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<tr>
<td>α-Tubulin</td>
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<td><img src="image6" alt="Image" /></td>
<td><img src="image7" alt="Image" /></td>
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</tr>
</tbody>
</table>

40 kD

50 kD

B.

Scr. siRNA

RAD51C siRNA

C.

Avg. no. of invading cells / 200X field of microscope

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<th>RAD51C siRNA</th>
</tr>
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<tbody>
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<td>n=2</td>
</tr>
<tr>
<td>Avg. no.</td>
<td>100</td>
<td>450</td>
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Bar chart showing a significant increase in the average number of invading cells after treatment with RAD51C siRNA compared to Scr. siRNA.
4.10. Effect of RAD51C downregulation on XRCC3 protein levels:

The CX3 complex is a result of XRCC3 and RAD51C interaction and downregulation of either partner has been known to directly lower the levels of the other, in other cell types\textsuperscript{26}. Since we also observed RAD51C to be dependent on XRCC3 for its stability in T4-2 cells, we wanted to ask the reciprocal question of whether XRCC3 depended on RAD51C for its stability. For this, T4-2 cells were transfected with RAD51C targeting or scrambled control siRNAs and cultured for 48hrs. si-RAD51C lysates were probed for XRCC3 protein levels in order to determine whether T4-2 cells show a similar co-regulation of XRCC3 and RAD51C as has been previously reported.

Results from the band densitometric analysis show a 60% reduction in the levels of XRCC3 protein, when RAD51C was downregulated 52\% (Figure 4.13)
Figure 4.13: Effect of RAD51C downregulation on XRCC3 protein levels: Left panel: RAD51C was downregulated using RAD51C siRNA and checked for downregulation of RAD51C levels. The same lysates were probed for XRCC3 protein. α-tubulin was used as loading control. Right panel: Quantified band densitometric values of respective proteins from si-RAD51C lysates (protein / loading control) normalized to scrambled siRNA values, for one experiment.
4.11: **Effect of XRCC3 siRNAs on the ability of T4-2 conditioned medium to induce invasiveness in T4-2 cells:**

Conditioned medium (CM) from XRCC3 siRNA transfected T4-2 cells was collected after 48 hrs of siRNA treatment and invasion assays were performed using just T4-2 cells with 50% conditioned medium, to determine whether it has any secreted factors that assist in invasion. Two separate experiments were performed using 25%CM, 50%CM, 75%CM from XRCC3 siRNA-1, XRCC3 siRNA-2, and scrambled control siRNA transfected T4-2 cells. Lysates were prepared from the siRNA transfected cells to check for XRCC3 protein downregulation, and conditioned medium from each sample was collected and used in an invasion assay with T4-2 cells in 25%, 50% and 75% concentrations. Statistics were performed the same way as for normal invasion assays.

Results show insignificant P values for 25%CM and 50% CM for XRCC3 siRNA-1 and -2 vs. scrambled siRNA due to large deviations in invasion assay counts, across experiments (n=2), although a significant increase in invasion trends was observed in both of the 2 experiments, exclusively. Results show a significant increase in T4-2 cell invasiveness with 75%CM from both XRCC3 siRNAs vs. scrambled siRNA. These findings suggest a direct effect of XRCC3 siRNAs on the ability of T4-2 CM to induce invasiveness in T4-2 cells.
**Figure 4.14: Effect of XRCC3 siRNA on the ability of T4-2 conditioned medium to induce invasiveness in T4-2 cells (secreted factors):** (page 51) Three graphs representing invasion assay results of T4-2 cells treated with 25%CM, 50%CM and 75%CM, respectively, each collected from scrambled control siRNA (black), XRCC3 siRNA-1 (red), and XRCC3 siRNA-2 (green) transfected T4-2 cells from 2 sets of experiments (n=2). Mean values (Avg. no. of invading cells / 200X field / well; triplicate wells) of 25%CM, 50%CM and 75%CMs of scrambled control siRNA from each experiment were normalized to 1 and the respective XRCC3 siRNA mean values were divided by scrambled siRNA mean values to obtain normalized averages. x-axis of the graphs were plotted with normalized average values for 25%CM, 50%CM, 75%CM, each collected from scrambled control siRNA vs. 2 XRCC3 siRNA transfected T4-2 cells. Standard deviation across normalized average values of each sample was plotted as y-error bars. y-axis represents fold difference with respect to scrambled siRNA values, normalized to 1. Two experiments. t-test , P values- For 25% CM: P= 0.307 (XRCC3 siRNA-1 vs. scr. siRNA) and P= 0.267 (XRCC3 siRNA-2 vs. scr. siRNA); For 50% CM: P= 0.410 (XRCC3 siRNA-1 vs. scr. siRNA) and P= 0.418 (XRCC3 siRNA-2 vs. scr. siRNA); For 75%CM: P= 1.99777E-06 (XRCC3 siRNA-1 vs. scr. siRNA) and P= 0.0188 (XRCC3 siRNA-2 vs. scr. siRNA).
4.12. Effect of XRCC3 siRNA on activation and localization of FAK:

To determine whether canonical adhesion pathways are downstream targets of XRCC3 in invasion, we asked whether it is involved in the phosphorylation of FAK and integrin-mediated invasion through IrBM.

Following siRNA mediated downregulation of XRCC3, protein levels of total FAK and FAK phosphorylated at tyrosine residue- 397 were monitored by western blotting. Furthermore, indirect immunofluorescence analysis was performed with the same antibodies, plus one antibody for FAK phosphorylated at tyrosine-861 residue, to monitor the actual localization and levels of these proteins on XRCC3 siRNA-1 and -2 transfected T4-2 cells vs. scrambled control siRNA transfected T4-2 cells. FAK and phospho-FAK containing adhesion complexes close to the cell surface were analyzed by confocal microscopy.

Results from the indirect immunofluorescence experiment show no difference in the expression and localization of total FAK in XRCC3 siRNA-1 and XRCC3 siRNA-2 transfected T4-2 cells as compared to scrambled siRNA (Figure 4.15). We were convinced that the FAK signal was specific since its amount increased as expected in response to EGF treatment (Figure 4.16). There was no difference among scrambled siRNA, XRCC3 siRNA-1 and XRCC3 siRNA-2 treated T4-2 cells’ total FAK signal, even in the presence of EGF. Western blotting on si-XRCC3 lysates showed no difference between protein levels of total FAK in scrambled control siRNA compared to XRCC3 siRNA transfected T4-2 cells (Figure 4.17).
The expression and localization of autophosphorylated FAK-Y397 residue increased in XRCC3 siRNA transfected T4-2 cells with a clear increase in signal intensity as compared to scrambled control siRNA (Figure 4.18). The FAK-Y397 signal specificity was verified by an increase in the punctuate signal after EGF+ TGFβ treatment (Figure 4.19). Interestingly, after induction of FAK-Y397 by these growth factors, there was no difference in expression between scrambled siRNA and XRCC3 siRNA transfected T4.2 cells. This suggested that EGF+ TGFβ activated signaling pathways that affect FAK-Y397 phosphorylation are downstream of XRCC3. Western Blotting showed no significant increase in total protein levels of FAK-Y397 (Figure 4.20), suggesting that the increased signal of FAK-Y397 did not require an increase in overall expression.

The focal adhesion localization of FAK-Y861 residue was also found to be increased in both XRCC3 siRNA treated T4-2 cells as compared to scrambled siRNA (Figure 4.21). Interestingly, treatment of cells with EGF abolished the difference of scrambled siRNA and XRCC3 siRNA treated cell expression of FAK-Y861 (Figure 4.22), suggesting that the upregulation of this phosphorylation event by XRCC3si was upstream of EGF mediated growth factor signaling.
Figure 4.15: Effect of XRCC3 siRNA on expression and localization of total FAK:
Confocal microscopy images above show the localization of total FAK in immunostained T4-2 cells. Each Alexa Fluor 488 (green signal) image at 100X magnification is followed by its composite DAPI+DIC merged view. Two Alexa fluor images (including their adjacent DAPI+DIC views) are shown for each of the 3 conditions: A. T4-2 cells treated with scrambled control siRNA; B. T4-2 cells treated with XRCC3 siRNA-1. C. T4-2 cells treated with XRCC3 siRNA-2.
Figure 4.16: Panel of total FAK controls: The images above show the localization of total FAK in immunostained T4-2 cells for negative and positive controls. Each Alexa Fluor (green signal) confocal image at 100X magnification is followed by its composite DAPI+DIC merged view. One Alexa fluor image (including its adjacent DAPI+DIC view) is shown for each of the following 4 conditions: A. T4-2 cells treated with anti-mouse secondary antibody only (negative control); B. T4-2 cells treated with scrambled control siRNA + growth factor EGF to maximize green signal output (positive control for scrambled siRNA); C. T4-2 cells treated with XRCC3 siRNA-1 + growth factor EGF (positive control for XRCC3 siRNA); D. T4-2 cells treated with XRCC3 siRNA-2 + growth factor EGF (positive control for XRCC3 siRNA-2).
Figure 4.17: Effect of XRCC3 siRNA on total-FAK protein levels: Western blot analysis of total FAK in scrambled siRNA transfected T4-2 cells vs. the 2 XRCC3 siRNA transfected T4-2 cells. Cell lysates were first probed for XRCC3 protein to check for downregulation of XRCC3 protein levels. Same lysates were further probed for total-FAK protein levels. Graph on the right side shows avg. band densitometric values of total-FAK levels normalized to their scrambled control siRNA values. 2 experiments, t-test, $P=0.097$ (XRCC3 siRNA-1 vs. scr. siRNA and $P=0.666$ (XRCC3 siRNA-2 vs. scr. siRNA).
Figure 4.18: Effect of XRCC3 siRNA on expression and localization of autophosphorylated FAK-Y397: Confocal microscopy images above show the expression and localization of autophosphorylated FAK-Y397 in immunostained T4-2 cells. Each Alexa Fluor 488 (green signal) image at 100X magnification is followed by its DAPI+DIC merged view. Two Alexa fluor images (including their adjacent DAPI+DIC views) are shown for each of the 3 conditions: **A.** T4-2 cells treated with scrambled siRNA; **B.** T4-2 cells treated with XRCC3 siRNA-1; **C.** T4-2 cells treated with XRCC3 siRNA-2.
Figure 4.19: FAK-Y397 expression in the presence of growth factors: Localization of the autophosphorylated FAK-Y397 in immunostained T4-2 cells treated with EGF + TGFβ. Each Alexa Fluor (green signal) confocal image at 100X magnification is followed by its composite DAPI+DIC merged view. Two Alexa fluor images are shown for each of the 3 conditions: A. T4-2 cells treated with scrambled control siRNA + growth factors EGF and TGFβ (Positive control for scrambled siRNA); B. T4-2 cells treated with XRCC3 siRNA-1+ growth factors EGF+ TGFβ (Positive control for siRNA-1); C. T4-2 cells treated with XRCC3 siRNA-2 + growth factors EGF and TGFβ (Positive control for XRCC3 siRNA-2).
**Figure 4.20: Effect of XRCC3 siRNA on FAK-Y397 protein levels:** Western blot analysis of FAK-Y397 residue in scrambled siRNA transfected T4-2 cells vs the 2 XRCC3 siRNA transfected T4-2 cells. Cell lysates were first probed for XRCC3 protein to check for downregulation of XRCC3 protein levels. Same lysates were further probed for FAK-Y397 protein levels. Graph on the right side shows avg. band densitometric values of FAK-Y397 levels in XRCC3 siRNA-1 and XRCC3 siRNA-2 transfected T4-2 lysates normalized to scrambled siRNA values. 4 experiments, t-test, $P=0.674$ (XRCC3 siRNA-1 vs. scr.siRNA) and $P=0.733$ (XRCC3 siRNA-2 vs. scr.siRNA).
Figure 4.21: Effect of XRCC3 siRNA on expression and localization of FAK-Y861:
Confocal microscopy images above showing the localization of FAK-Y861 in immunostained T4-2 cells. Each Alexa Fluor 488 (green signal) image at 100X magnification is followed by its composite DAPI+DIC merged view. Two Alexa fluor images (including their adjacent DAPI+DIC views) are shown for each of the 3 conditions: **A.** T4-2 cells treated with scrambled control siRNA; **B.** T4-2 cells treated with XRCC3 siRNA-1; **C.** T4-2 cells treated with XRCC3 siRNA-2.
Figure 4.22: Panel of FAK-Y861 controls: Localization of FAK-Y861 in immunostained T4-2 cells for negative and positive controls. Each Alexa Fluor (green signal) image at 100X magnification is followed by its DAPI+DIC merged view. One Alexa fluor image is shown for a and b each and two Alexa Fluor images are shown for C and D each. A. T4-2 cells treated with anti-rabbit secondary antibody only (negative control); B. T4-2 cells treated with scrambled siRNA+ growth factor EGF (positive control for scrambled siRNA); C. T4-2 cells treated with XRCC3 siRNA-1 + growth factor EGF (positive control for XRCC3 siRNA-1); D. T4-2 cells treated with si-XRCC3 Ambion siRNA+ growth factor EGF (positive control for XRCC3 siRNA-2).
4.13: **Effect of XRCC3 siRNAs on cell-surface beta-1 integrin levels:**

Previously, PLK1 was found to regulate invasion via a mechanism of phosphorylating vimentin which in turn controls the cell surface levels of β1 integrin. To determine if XRCC3 regulates invasion via a similar pathway, we wanted to see if XRCC3 siRNAs have any effect on the total expression and cell surface levels of total beta-1 integrin and activated beta-1 integrin. For this, T4-2 cells were transfected with control or XRCC3 siRNAs, downregulation was confirmed by western blot, and the beta1 integrin levels were determined by FACS analysis of cells that were immunostained for XRCC3 without being permeabilized.

Results show no significant difference in the percentage of cells expressing the proteins or the expression levels total beta-1 integrin or activated beta-1 integrin in XRCC3 siRNA transfected T4-2 cells as compared to scrambled control siRNA on the cell surface (Figure 4.23). It is possible that some other combination of α-β- integrins could be affected by XRCC3 siRNAs. However, the XRCC3 regulation of invasion appears to be independent of altering beta1 integrin, rendering it a pathway different than that observed for PLK1 previously.
Figure 4.23: Effect of XRCC3 downregulation on cell-surface beta-1 integrin levels: Percentage gated (for intact cells) positive cells for each siRNA type are plotted followed by the graph of their averaged X-Median values of gated positive cells for A. total beta-1 integrin and B. activated beta-1 integrin, from 3 sets of experiments. In each experiment, mean value (X-median values; triplicate samples) for scrambled siRNA was normalized to 1, XRCC3 siRNA-1 and siRNA-2 mean values were divided by scr.siRNA mean values to get normalized average values which were used to plot the graph. Standard deviations of normalized average values from each sample were used to plot y-error bars. 3 experiments, t-test, P values: For total beta-1 integrin, $P= 0.367$ (XRCC3 siRNA-1 vs. scr. siRNA) and $P= 0.797$ (XRCC3 siRNA-2 vs. scr. siRNA); For Activated beta-1 integrin: $P= 0.517$ (XRCC3 siRNA-1 vs. scr. siRNA) and $P= 0.319$ (XRCC3 siRNA-2 vs. scr.siRNA).
5. **Discussion:**

**XRCC3 as a negative regulator of invasion:**

Previous results showed that overexpression of XRCC3 in T4-2 downregulated invasion. Since this downregulation was accompanied by a decrease in cell number as well, we wanted to determine whether siRNA downregulation of XRCC3 would provide a more direct link to invasion. In order to verify the role of XRCC3 in invasiveness, we tested the effect of downregulating XRCC3 on the invasiveness of T4-2 cells. The invasion assay results show a significant increase in invasiveness when XRCC3 was downregulated. Furthermore, during the invasion assay time span of 48 hours, results from the MTT assay show no significant change in the growth and viability of cells when treated with XRCC3 siRNAs as compared to scrambled siRNA. Parallel to this, results from the TUNEL apoptosis assay show no significant change in number of apoptotic dead cells in XRCC3 siRNA treated T4-2 cells. These results support a direct role for XRCC3 as a negative downregulator of invasion.

**Involvement of another RAD51 paralog RAD51C in Invasion:**

XRCC3 being a DNA repair gene, acts in concert with RAD51 paralogs B, C, D and XRCC2 to repair DNA breaks. To see if these interactions are important in terms of invasion, as they have been demonstrated for DNA repair, we studied the effect of XRCC3 downregulation on the levels of RAD51 paralogs. It has been demonstrated that proteins that form complexes to function together may be subjected to regulatory pathways that co-regulate their levels in the cell. In addition, removing one component
of a complex of proteins may signal proteolysis of the rest of the proteins in the complex which function together. We wanted to see if downregulating XRCC3 signal changes in the synthesis or destruction regulatory mechanisms in the cell to have any effect on the levels of RAD51 paralogs, especially RAD51C with which it forms the CX3 complex. Our results confirmed that downregulation of XRCC3 significantly reduces the levels of RAD51C, but not RAD51B or RAD51D in invasive T4-2 cells. Since RAD51C levels dropped with XRCC3 downregulation, we decided to see if it had any parallel effect on invasion. siRNA mediated downregulation of RAD51C in T4-2 cells lead to a significant increase in the number of invading cells. As RAD51C and XRCC3 form the CX3 complex and are dependent on each other for their stability, we checked for levels of XRCC3 protein in RAD51C siRNA transfected T4-2 cells. As anticipated and proven before in HT1080 and HeLa cell lines\textsuperscript{26}, results showed lower levels of XRCC3 in RAD51C depleted T4-2 cells. Invasion assay results from individual knockdown of XRCC3 and RAD51C show an increase in invasiveness when either of the two proteins is downregulated. One of our future experiments would be to simultaneously downregulate both RAD51C and XRCC3 using siRNAs and see if there is any collaborative effect on invasion. In addition, mutations affecting the interaction of XRCC3 and RAD51C could answer the question of whether this is a functionally significant interaction.

**Effect of XRCC3 downregulation on canonical invasion pathways:**

Invasion is considered to be a multifactorial process involving a complex and dynamic interaction of a variety of proteolytic enzymes, protease inhibitors, growth factors, cell
dissociating cytokines, cell-cell and cell-substrate adhesion molecules. The mechanism of invasion can be characterized into two— the first category consisting of cell surface and secreted proteins such as adhesion receptors, degradative enzymes and their inhibitors, and motility-stimulating cytokines; and the second category consisting of regulatory proteins and pathways inside the cell such as tyrosine phosphorylation events, calcium-mediated signaling and G-protein activation.

To understand the mechanism of invasion followed by XRCC3, we performed invasion assays with conditioned medium from XRCC3 siRNA transfected T4-2 cells to check whether the CM contains any secreted factors that could assist or increase the invading capacity of T4-2 cells. Our results showed increased invasion of T4-2 cells when treated with CM from XRCC3 siRNA transfected T4-2 cells. One of the future experiments would be to precipitate proteins from the conditioned medium, and do mass spectrometry analysis for identification of suitable candidates known to be involved in invasion. Previously, the membrane form of another double strand break repair protein-Ku heterodimer (involved in non-homologous end-joining repair) was found to associate with MMP9 at the cell surface of leukemic cells and was found to be involved in degradation of ECM components. Since microarray analysis of HMT-3522 cell line showed elevated and functional expression of MMP9, 13, 15 and 17 in invasive T4-2 cells than pre-invasive cells, all these matrix proteases could be possible candidates to look for, in terms of secreted factors that assist in invasion.

Increased FAK expression and its tyrosine phosphorylations in malignant cells have been shown to have a good correlation with the progression to an invasive cell
phenotype\textsuperscript{41}. FAK is also essential for oncogenic transformation and cell invasion in SKBR-3, T47D, MCF-7, and MDA-231 invasive breast cancer cells. FAK phosphorylation at Tyr-397, -861, and -925 residues have been shown to promote cell invasion\textsuperscript{42}. Integrin-stimulated FAK phosphorylation at Y397 promotes the Src homology domain 2 (SH2)-dependent binding of Src family tyrosine kinases and the formation of an activated FAK–Src complex. FAK–Src signaling has also been shown to synchronize MMP-mediated extracellular matrix proteolysis and cell motility thereby facilitating an invasive phenotype. Following ligand binding to integrins or growth factor receptors, FAK-Y397 autophosphorylation leads to the formation of the FAK-Src-Cas/Crk-DOCK180 complex, increased Rac activation, increased matrix metalloproteinase-9 (MMP) expression and activity, formation of lamellipodia, and ultimately an increase in cell migration\textsuperscript{43, 44}.

In another recent study, phosphorylation of focal adhesion kinase was shown to promote extravasation in AU-565 and MDA-MB-231 metastatic breast cancer cell lines via Y397, Y861 and Y925 phosphorylations. Our results showed that total FAK localization did not change total FAK but Y397 and Y861 phosphorylated FAK formed larger number of FAK complexes when XRCC3 was downregulated. This is consistent with a role for Y397 and Y861 phosphorylations of FAK in modulating the positive effect of XRCC3 downregulation on invasion. Interestingly, XRCC3 inhibitions were upstream of growth factor signaling pathways since stimulating cells with EGF and TGFβ abolished the upregulation effect of XRCC3-si on Y397 and Y861 phosphorylations at focal adhesions i.e, once growth factors induce focal adhesion formation and activation, there is no additional effect of XRCC3.
Based on these results, we propose the following model for how XRCC3 acts as a negative regulator of invasion.

**Figure 5.1: Model for XRCC3 as a negative regulator of invasion:** Downregulating XRCC3 or its loss during cancer progression increases cell invasion via upregulating focal adhesion formation and activation. XRCC3 is upstream of EGF / TGFβ mediated growth factor pathways for inducing focal adhesion mediated cell invasion. Secreted factors are also affected by XRCC3, potentially upregulating MMPs such as MMP9, 13, 15 and 17. These MMPs could act upstream or downstream of EGF / TGFβ and FAK activation to induce invasion.
Overall these results assign a new function for XRCC3 as a negative regulator of invasion in breast epithelial cells. As a DNA double-strand break repair protein, it joins others, such as Ku80 that is also involved in DNA double-strand break repair, as well as PLK1, which acts as a modulator of genome stability via its effects on cell cycle arrest in response to DNA damaging agents. Additional candidate invasion modulators of similar function include Mre11 / RAD50 / NBS1 that also function in double-strand break repair, as well as the centromeric protein CENPA (Rizki laboratory, unpublished). Although there is no apparent similarity in their protein domains or known functions, their effect on the invasiveness of breast cells suggests that they may all plug into similar pathways. The presence of proteins such as XRCC3 (and the MRN complex), which preserve genome integrity as well as prevent invasion make sense, since both functions help maintain normalcy and avoid tumorigenic transformation. Although the details of their mechanisms will continue to be deciphered, the existence of these dual function GISEM genes produce a clan that could be clinically valuable to therapeutic targeting as well as biomarker studies since genome instability and invasiveness are major functions that can be targeted or monitored. The expectation is that XRCC3 and other GISEM genes will provide clinically relevant targets and biomarkers of risk of breast cancer progression.
6. References:


