

Ionizing radiation predisposes non-malignant human mammary epithelial cells to undergo TGF β - induced epithelial to mesenchymal transition

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Abstract

Transforming growth factor β 1 (TGF β) is a tumor suppressor during the initial stage of tumorigenesis, but it can switch to a tumor promoter during neoplastic progression. Ionizing radiation (IR), both a carcinogen and a therapeutic agent, induces TGF β activation *in vivo*. We now show that IR sensitizes human mammary epithelial cells (HMEC) to undergo TGF β -mediated epithelial to mesenchymal transition (EMT). Non-malignant HMEC (MCF10A, HMT3522 S1 and 184v) were irradiated with 2 Gy shortly after attachment in monolayer culture, or treated with a low concentration of TGF β (0.4 ng/ml), or double-treated. All double-treated (IR+TGF β) HMEC underwent a morphological shift from cuboidal to spindle-shaped. This phenotype was accompanied by decreased expression of epithelial markers E-cadherin, β -catenin and ZO-1, remodeling of the actin cytoskeleton, and increased expression of mesenchymal markers N-cadherin, fibronectin and vimentin. Furthermore, double-treatment increased cell motility, promoted invasion and disrupted acinar morphogenesis of cells subsequently plated in MatrigelTM. Neither radiation nor TGF β alone elicited EMT, even though IR increased chronic TGF β signaling and activity. Gene expression profiling revealed that double treated cells exhibit a specific 10-gene signature associated with Erk/MAPK signaling. We hypothesized that IR-induced MAPK activation primes non-malignant HMEC to undergo TGF β -mediated EMT. Consistent with this, Erk phosphorylation were transiently induced by irradiation, persisted in irradiated cells treated with TGF β , and treatment with U0126, a Mek inhibitor, blocked the EMT phenotype. Together, these data demonstrate that the interactions between radiation-induced signaling pathways elicit heritable phenotypes that could contribute to neoplastic progression.

Introduction

An emerging concept in cancer biology is that carcinogens can compromise tissue integrity by eliciting altered phenotypes and tissue composition (reviewed in (1)). Intercellular and extracellular signals are critical to the suppression of neoplastic growth while disruption of cell–cell and cell-matrix interactions is implicated, if not required, for neoplastic progression. Indeed, reversion of the malignant phenotype by modulating extracellular signals suggests that cancer cells are susceptible to signals from the microenvironment (2).

We have postulated that ionizing radiation (IR) alters cell phenotypes, that in turn contribute, directly or indirectly, to carcinogenesis (3). IR activates multiple signaling pathways depending on the cell type, radiation dose and cell status (reviewed in (4)). IR also affects the activity or abundance of proteases, growth factors, cytokines, and adhesion proteins that are involved in tissue remodeling (reviewed in (5)). We have shown that transforming growth factor β 1 (TGF β) is activated following IR, and that it in turn mediates cellular and tissue radiation responses (6, 7). Although TGF β is considered to be a potent tumor suppressor during the initial stage of tumorigenesis, principally through its ability to cause growth arrest and apoptosis, numerous reports show that TGF β can switch to tumor promoter during neoplastic progression (reviewed in (8)). We have shown that the progeny of irradiated non-malignant human mammary epithelial cells (HMEC) cultured with TGF β exhibit compromised morphogenesis, polarity and growth control when cultured in reconstituted basement membrane (9).

Some epithelial tumors, particularly those that overexpress TGF β (10), exhibit mesenchymal characteristics and are more aggressive. Several lines of evidence have led researchers to link this morphological shift during carcinogenesis to the physiological process of epithelial to mesenchymal transition (EMT). EMT is characterized by loss of epithelial cell polarity, loss of cell-cell contacts, acquisition of mesenchymal markers and phenotypic traits that include increased cell motility (reviewed in (11)). Although the clinical relevance of EMT in late-stage tumor progression is controversial, it is generally agreed that EMT can occur during cancer progression (11). Approximately 18% of breast cancers exhibit evidence of EMT (12). EMT has recently been reported during tumor recurrence after therapy (13). TGF β has been particularly targeted as a mediator of EMT during neoplastic progression, but analysis of normal epithelial and cancer cell lines showed that TGF β alone rarely induces EMT by (14).

In the present studies, we found that a single exposure to IR sensitizes HMEC to undergo TGF β -mediated EMT and exhibit all the classic hallmarks of EMT. Neither irradiation nor TGF β alone was sufficient to elicit EMT even though TGF β activity is elevated in irradiated cells. Gene expression profiling revealed a specific signature in double-treated cells associated with MAPK signaling. As previously reported, IR induces transient activation of the MAPK pathway, but exposure to low concentrations of TGF β maintains this pathway activity, which is required for maintenance of EMT. We postulate that the pathway signaling interactions between radiation-induced MAPK and TGF β elicit the heritable EMT phenotype.

Materials and Methods

Cell culture: HMEC were cultured in serum free medium as previously described for HMT-3522 S1 (S1; passages 55-60) (15), MCF10A (ATCC, Manassas,VA), 184 HMEC (184v; passage 7-10) (16) and HMT-3522-S2 (S2) (17). Recombinant human TGF β (0.4 ng/ml) was added at the time of plating or of irradiation. HMEC were irradiated 4-5h (S1, MCF10A and S2) or 10-14h (184v HMEC) post plating using either 160 KV X-ray or ^{60}Co γ -radiation with a total dose of 2Gy. Control plates were sham irradiated. In some experiments, medium was supplemented with 10 $\mu\text{g/ml}$ TGF β pan-specific neutralizing antibody (R&D Systems, Minneapolis, MN) or with an equivalent concentration of non-specific mouse IgG. When noted, some cultures were treated with 10 μm U0126 (Cell Signaling, Beverly, MA) or DMSO.

Reagents: Antibodies to phosphorylated Erk1/2 (Thr202/Tyr204), phosphorylated MEK (Ser217/221), Erk1/2 and Mek1/2 were from Cell Signaling. Phalloidin was from Molecular Probes (Eugene, OR). E-cadherin, β -catenin and N-cadherin antibodies were purchased from BD Biosciences (San Jose, CA) and vimentin-clone VIM 13.2, β -actin and fibronectin antibodies were from Sigma (St. Louis, MO). ZO-1 antibody was obtained from Zymed (South San Francisco, CA). SMAD 2/3 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Recombinant TGF β and TGF β pan-specific neutralizing antibody were obtained from R&D Systems.

Immunofluorescence: Cells grown on LabTek 8-well chamber slides; fixed with 80% methanol for 10 min (N-cadherin, ZO-1) or methanol:acetone (vimentin, β -catenin) or 4% paraformaldehyde (fibronectin, SMAD3, phalloidin) followed by treatment with 0.1% Triton (N-cadherin, SMAD3) for 10 min. For E-cadherin staining cells were either fixed with 80% methanol or extracted with CSK buffer (15), followed by 4% paraformaldehyde fixation. Nuclei were counterstained with DAPI (4',6-Diamidino-2-Phenylindole) using 0.5 ng/ml.

TGF β Bioassay: Mink lung cells transfected with the promoter of plasminogen activator inhibitor linked to a luciferase reporter (PAI-L) were used to analyze TGF β in the conditioned media as previously reported (18).

Protein analysis: Cells were lysed as previously described (15) or in a buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5 mM MgCl $_2$, 0.2 mM EGTA, 1% Triton X-100, protease inhibitor mixture (20 $\mu\text{l/ml}$; Sigma), 1 mM Pefabloc and 50mM glycerol-2-phosphate (Sigma) or phosphatase inhibitor cocktail set II (Calbiochem, San Diego, CA). Extraction and immunoprecipitation of soluble and insoluble pools of E-cadherin was done as previously described (19). Proteins were separated on 4-15% SDS-PAGE gels and transferred to Immobilon-P (Millipore, Billerica, MA) or nitrocellulose (Amersham Lifescience); and probed with indicated primary antibodies. In some instances, detection was accomplished using chemiluminescence of secondary antibodies labeled with horseradish peroxidase followed by densitometry analysis of films. Alternatively, detection was performed using the Odyssey system (LI-COR, Lincoln, NE) as previously described (20). Target proteins were normalized to β -actin for loading.

Motility and invasion assays: Confluent HMT3522 S1 or MCF10A cultures were grown in medium containing EGF. The cultures were scratched with a pipette tip and washed to

remove detached cells. The invasion assay was performed as previously described (21). The number of invading cells was the average of 3 wells from 2 duplicate experiments.

RNA purification: Cells were washed with PBS; denatured in Trizol; scrapped and subjected to chloroform extraction. After centrifugation, the upper phase was precipitated with an equal volume of isopropanol. RNA precipitates were resuspended in RNase free water and further purified on RNeasy columns (Qiagen, Germany). RNA quality was assessed on an Agilent Bio-Analyzer. The dataset analyzed by microarray included biological duplicates for each treatment in two independent experiments and three sham-treated samples.

Microarray processing and analysis: Cy3/Cy5-labeled cDNAs were generated from RNA and hybridized to HG-U133A chips (Affymetrix Inc.,) at the Lawrence Berkeley National Laboratory HTArray Core Facility (<http://hta.lbl.gov>). Samples were analyzed and clustered with the (UNO) One Color GenetrafficTM software version 3.2-12 (Iobion Informatics LLC, Stratagene, La Jolla, CA). Genes whose expression was specifically altered by treatment were defined as those in which Cy5/Cy3 ratio was more than 1.75 fold ($\text{lmean log}_2\text{ratio} > 0.8$) from baseline in at least three out of the four treated samples compared to the three sham samples. Significance analysis tests ($p < 0.05$) were performed using Excel between sham samples and either IR, TGF β or TGF β +IR samples.

Real-time polymerase chain reaction (PCR): Total RNAs were pre-treated with amplification grade DNase I (Invitrogen, Carlsbad, CA) then primed with random hexamers to generate cDNAs using a SuperscriptTM III first-strand synthesis kit according to the manufacturer's instructions (Invitrogen). 1 μ l of non diluted cDNAs were then amplified with Lightcycler FastStart DNA master SYBR Green I (Roche Applied Science, Mannheim, Germany) using a Light Cycler (Roche Applied Science, Mannheim, Germany). TCF8, FGF2 and CDH1 specific primers optimized for SYBR Green real-time PCR were purchased from SuperArray Bioscience Corporation (Frederick, MD).

Image acquisition, processing and analysis: Imaging was done as previously described (20). Colony segmentation from phase images was done using live wire tool from MIPAV (22). Area and shape factors were calculated using Matlab (MathWorks Inc, Natick, MA) and DIPimage (image processing toolbox for Matlab, Delft University of Technology, The Netherlands).

Results

The progeny of irradiated HMEC undergo EMT in response to TGF β

We previously reported that E-cadherin immunofluorescence significantly decreased in double-treated HMT3522 S1 cultured in Matrigel compared to control or single-treated cells and failed to establish acinar morphology typical of HMEC (9). EMT is one means of disrupting morphogenesis, e.g. during wound healing. To examine the possibility that EMT underlies the response to radiation and TGF β , we compared the epithelial characteristics of HMEC in traditional monolayer culture. HMEC were irradiated with 2 Gy shortly after attachment, or treated with low concentrations of TGF β (400 pg/ml), or double-treated, and cultured to approximately 80% confluence. As observed previously, E-cadherin and β -catenin immunolocalization in monolayer HMEC was significantly reduced in double-treated cells (Figure 1A). The function of an epithelium as a barrier requires the establishment of tight junctions, evident by lateral, apical localization of ZO-1. ZO-1 immunofluorescence showed distinct punctate localization at cell borders of control and irradiated cells. TGF β treatment somewhat altered ZO-1 staining, such that it appeared as a coarser, punctate-like pattern at the cell boundaries (Figure 1A). However, ZO-1 staining completely disappeared in double-treated cells.

Notably, double treatment resulted in spindle shaped, elongated cells, a phenotype which was examined by phalloidin staining of actin. Actin localized mainly at cell-cell borders of control and irradiated HMEC with very few stress fibers. TGF β alone elicited a mild change in actin organization. Double-treated cells displayed dramatic actin reorganization consisting of longitudinal stress fibers, characteristic of fibroblasts (Figure 1A).

In light of the loss of epithelial markers in irradiated HMEC cultured with TGF β , we investigated whether there was a concomitant gain of mesenchymal markers, fibronectin, vimentin and N-cadherin (Figure 1B). Immunofluorescence analysis of the double-treated group revealed increased fibronectin deposition compared to sham and single-treated cells. Accumulated fibronectin was arrayed perpendicular to the cell edge and in randomly oriented networks. The intermediate filament protein vimentin dramatically increased following double treatment. Consistent with the shift to an elongated morphology, vimentin filaments were organized in a longitudinal meshwork. N-cadherin is normally present mainly in neuronal and muscle tissues, but is aberrantly expressed in epithelial tumors where it has been proposed to promote migration and invasiveness of carcinoma cells (23). N-cadherin immunofluorescence was dramatic increased following double treatment in a pattern reciprocal to E-cadherin loss.

Loss of epithelial markers and gain of mesenchymal markers in double-treated cells was accompanied by epithelial to mesenchymal cell shape change. Phase microscopy HMT3522 S1, MCF10A and 184v HMEC (Figure 1C) showed that untreated HMEC and irradiated cells displayed typical cuboidal appearance of epithelial cells. TGF β elicited a modest shape change, particularly in 184v HMEC. In contrast, the morphology of all three HMEC following double-treatment shifted to spindle-shaped. The morphological response was observed even when TGF β was added 48h post-IR, and was not reversible upon removal of TGF β from the culture media for 48h (data not shown). Taken together, the concomitant increased expression of mesenchymal markers, loss of epithelial markers

and morphological response of the progeny of irradiated HMEC to TGF β suggest that cells have undergone EMT.

TGF β alters the cytoskeletal associated E-cadherin/ β -catenin complexes in irradiated HMEC

Although E-cadherin immunofluorescence was significantly reduced only in double-treated cells (Figure 1A), western blot analysis of total E-cadherin protein expression levels were comparable in TGF β -treated and double-treated groups (Figure 2A). β -catenin protein levels followed the same pattern. This observation suggested that immunofluorescence was a function of protein localization, rather than abundance. In epithelial cells, E-cadherin and β -catenin are associated with the cytoskeleton at intercellular junctions that are resistant to detergent extraction (24). We tested whether the difference between immunofluorescence and immunoblotting was due to solubility by using differential detergent extraction followed by E-cadherin immunoprecipitation. Although TGF β reduced the soluble pool of E-cadherin regardless of irradiation, the insoluble, cytoskeletal associated E-cadherin was significantly decreased only in double-treated cells (Figure 2B). The distribution of β -catenin was similarly affected. Thus, TGF β alters the cytoskeletal associated E-cadherin/ β -catenin complexes only in irradiated HMEC.

Functional consequence of the response of irradiated HMEC to TGF β

During physiological processes and in cancer, EMT is often accompanied by acquisition of cell motility and invasiveness (11). We analyzed the migratory properties of double-treated HMEC using a monolayer scratch assay and time-lapse videomicroscopy (Figure 3A). Control or irradiated cells moved as a sheet evidenced by a smooth edge and failed to fill the gap during the 40h observation period. Consistent with their intermediate cytoskeletal and cell-cell adhesion phenotype, TGF β -treated HMEC began to close the wound after 20h, but the cells still maintained sheet formation and by 40h had not closed the gap. In contrast, irradiated HMEC cultured with TGF β moved as single, spindle-shaped cells and closed the gap by 40h by forming multicellular bridges of spindle shaped cells between the two areas. Thus the tissue-specific requirement for epithelia to maintain cell interactions as a sheet of cohesive cells is disrupted when irradiated HMEC are exposed to TGF β , which leads to independent single cell migration characteristic of mesenchymal cells.

The above observations led us to further investigate the invasive properties of HMEC by invasion assay through a membrane coated with Matrigel™, a basement membrane type matrix. HMT 3522 S1 did not invade in this assay under any condition. Therefore we used HMT 3522 S2, which are derived from further propagation of HMT 3522 S1 in EGF-free media (21). Cells were cultured as described above, then trypsinized and plated into the Boyden chamber assay in standard media. Few control HMT 3522 S2 cells were able to invade. The progeny of irradiated cells demonstrated greater invasion than treatment with TGF β alone when compared to the control. In contrast, irradiation of S2 cells and subsequent culture in the presence of TGF β increased the number of invading cells more than 100-fold compared to sham (Figure 3B).

There is ongoing discussion in the literature as to what criteria define true EMT *in vitro* and how to distinguish the EMT phenotype from a 'scattering' phenotype (11). Both phenotypes exhibit disruption of cell junctions, fibroblast-type morphology, and enhanced motility. However, it has been suggested that they can be distinguished by reversibility since the EMT phenotype, but not the scattering phenotype, persists after withdrawal of the stimulus (11). As mentioned above, TGF β withdrawal did not reverse the morphological shift, consistent with EMT. Since the progeny of irradiated HMEC cultured with TGF β exhibit disrupted morphogenesis in Matrigel™ (9), we used morphogenesis as a stringent test of persistence. Single and double-treated HMEC cells were trypsinized and replated in Matrigel™ for analysis of acinar morphogenesis without further TGF β stimulation. Replated double-treated cells formed larger colonies than control or single treated cells, which was measured by increased size and irregularity of colony shape (Figure 3C). We concluded that the phenotype elicited by IR and TGF β resulting in dysplastic morphogenesis (i.e. 3D growth deregulation and disorganization) and the persistence of these morphological alterations in the absence of additional TGF β is further evidence of EMT.

Irradiated HMEC produce active TGF β

Latent TGF β is activated in the irradiated mouse mammary gland *in vivo* (6), which could increase the susceptibility of irradiated cells to undergo TGF β mediated EMT by decreasing a threshold response. Nuclear translocation of receptor phosphorylated SMAD2 or 3 (R-SMAD) results from liganded TGF β receptor (8). Immunofluorescence analysis demonstrated that R-SMAD nuclear localization increased by 30% in irradiated HMEC 6 days after irradiation compared to control cells (Figure 4A). These data suggest that IR elicits persistent TGF β signaling. To determine whether IR increased TGF β production or activation, we analyzed the conditioned medium (CM) from irradiated cells using a TGF β bioassay (18). Bioassay of irradiated and control HMEC CM indicated similar levels of latent TGF β . Active TGF β , however, was undetectable in either CM (data not shown). Since the sensitivity of detection in CM for the bioassay is approximately 0.2 ng/ml, we reasoned that low levels of TGF β could be functional, although undetectable by bioassay. If so, then the CM should give rise to a similar phenotype in unirradiated HMEC seen in response to addition of exogenous TGF β . To investigate this hypothesis, we fed non-irradiated HMEC with 50% CM from either irradiated or non-irradiated HMEC cultures. CM from irradiated cells caused a significant decrease in E-cadherin protein levels when compared to cells treated with CM from non-irradiated cells. This response was reversed by adding TGF β neutralizing antibody to the cells grown in the presence of IR-treated CM (Figure 4B). To confirm that this biological activity produced by irradiated cells was indeed TGF β , irradiated cells were grown in the absence or presence of TGF β neutralizing antibody. When irradiated HMEC were grown in the presence of TGF β neutralizing antibody there was a significant reversal of the decrease in E-cadherin protein abundance compared to cells grown with control IgG control antibody (Figure 4C).

Based on the above data, we estimated that irradiated cultures produce approximately 100 pg/ml of active TGF β . We then tested whether EMT was a function of the additive effect

of exogenous (400 pg/ml) and endogenous TGF β (approximately 100 pg/ml) by treating HMEC with graded concentrations of TGF β . However, MCF10A treated with up to 1200 pg/ml TGF β did not undergo EMT (data not shown). Although IR induced TGF β activity in cultured HMEC, as it did in the mouse mammary gland (6), it was insufficient to disrupt HMEC acinar morphogenesis as shown in our previous study (9). Moreover, the progeny of irradiated HMT 3522 S1, MCF10A and 184v HMEC exhibited a modest response in terms of epithelial morphology, E-cadherin expression or invasion. Thus, chronic TGF β activation by irradiated HMEC is insufficient to drive the EMT phenotype.

Genetic programs underlying EMT in double-treated HMEC

In order to comprehensively describe the altered epithelial cell phenotype following double treatment, we compared transcript expression levels in sham, IR-, TGF β - and double-treated HMEC. The expression profile of the progeny of irradiated HMEC was similar to control HMEC. 43 genes were differentially expressed by TGF β -treated HMEC that included 28 up-regulated genes and 15 down-regulated genes with a >1.75-fold change in expression compared to sham samples (Supplementary Table). We identified 10 genes that constituted the double-treatment signature. Expression was significantly increased for five genes and decreased for the five other genes after double treatment compared to TGF β alone.

The five significantly up-regulated genes in double-treated samples were the growth factor FGF2/bFGF, the serine/cysteine proteinase inhibitor, SERPINA1, the interleukin 1 receptor-like protein, IL1RL1, the transcription factor TCF8/ZEB-1 and the zinc transporter SLC39A8 (Figure 5A and B). The five down-regulated genes in double-treated HMEC were the Wnt pathway transcription repressor secreted frizzled related protein 1 (SFRP1), the cysteine-rich protein, CRIP2, the aldo-keto reductase, AKR1C2, the cadherin EGF LAG seven-pass G-type receptor 2 (CELSR2), and the aminopeptidase O, C9orf3. Although transcript levels of five of these ten genes (SERPINA1, SLC39A8, CRIP2, AKR1C2 and CELSR2) were higher in TGF β -treated samples than in sham samples, they were below the 1.75-fold cut off used to define TGF β -responsive genes. Changes in gene expression in double-treated HMEC were validated for TCF8 and FGF2, by real-time RT-PCR (Figure 5B). For comparison, real-time RT-PCR of E-cadherin (CDH1) was decreased in both TGF β and double treated cells, consistent with the protein levels shown in Figure 2A.

The EMT signature genes have roles in cell-matrix interactions, cell motility/invasion, inflammation and disruption of adherens junctions. The products of TCF8/ZEB1 and FGF2 have been described to repress E-cadherin transcription and to promote EMT (25, 26). Some of the TGF β -responsive and EMT-associated signature genes identified here have been previously described (27, 28). An extensive bibliography search showed that 5 of the 10 EMT signature genes are directly or indirectly associated with the Erk/MAPK pathway, suggesting a specific functional role of Erk/MAPK activation in inducing EMT upon double-treatment. Several reports suggest that an independent stimulus must synergistically proceed or at least accompany the induction of EMT. The multiple

downstream effectors of Ras, such as MAPK, may be a requirement to induce a pre-malignant state and endow epithelial cells with an increased rate of proliferation (29).

Persistent activation of Erk/MAPK in double-treated HMEC

Consequently, we examined the possible involvement of the Erk/MAPK signaling cascade in the mediation of TGF β -induced EMT in irradiated HMEC by determining the activation of Erk1/2 and upstream activators, Mek1/2. The total amounts of Erk or Mek were similar following all treatments. Phosphorylated-Erk1/2 was modestly induced by TGF β but was significantly increased in irradiated HMEC treated with TGF β (Figure 6A, top panel). Consistent with this, Mek1/2 activation, as indicated by phosphorylation, was also increased upon double treatment.

There is a significant body of literature that suggests that Erk activation is required to initiate the EMT program (30, 31). IR can induce a rapid, ligand-independent activation of Erk/MAPK in cancer cells (32). To investigate whether activation of Erk1/2 by IR or TGF β *versus* double treatment was indeed instrumental in driving EMT, we monitored Mek/Erk activation at early time points (4-48h post IR). At 8h, Erk1/2 phosphorylation was significantly induced by IR when compared to TGF β or double-treatment. From 12-24h, MAPK pathway activation increased following IR or TGF β or double treatment compared to controls. However, at 48h post-IR, double-treated cells exhibited greater Erk activation compared to control and to single-treated HMEC (Figure 6, bottom panel).

To determine if persistent Erk activation was required for the EMT phenotype in double-treated HMEC, we used U0126, a specific Mek small molecule inhibitor. Treatment with U0126 dramatically decreased Erk activation in HMEC (Figure 6B). Treatment with U0126 also restored E-cadherin and cytoskeletal rearrangement (Figure 6C) and reduced cell migration in the wound closure assay (Figure 6D). These data suggest that IR and TGF β collaborate to increase Erk activation, which is necessary for establishment and maintenance of EMT (7 days). We propose that transient IR-induced Erk activation is sustained in the presence of additional TGF β , and that this event predisposes non-malignant HMEC to undergo TGF β -mediated EMT.

Discussion

Here we report that the progeny of irradiated HMEC are dramatically sensitized to undergo TGF β -induced EMT. IR and TGF β cooperated to induce a phenotypic transition that occurred in the progeny of cells irradiated once and persisted even in the absence of TGF β . This resulted in increased motility, enhanced invasion, disrupted epithelial morphogenesis, and was accompanied by a distinct pattern of gene expression.

TGF β has long been considered as both a positive and a negative effector of mammary tumorigenesis, acting early as a tumor suppressor but later as a stimulator of tumor invasion (8). Overexpression of constitutively active TGF β can induce EMT during tumor progression *in vivo* (33) and the overexpression of TGF β has been associated with poor prognosis of many human cancers (8). The phenotypes of breast cancer micrometastases in lymph nodes and the bone marrow have been interpreted as evidence that EMT occurs in primary tumors (34). However *in vivo* verification of EMT has been controversial, perhaps due to the transient and reversible nature of the process and to the lack of analytical tools that distinguish carcinoma cells undergoing EMT from neighboring stromal fibroblasts.

In vitro studies of EMT were originally described in cells of murine origin, frequently containing *ras* mutations. It has been reported in a very limited number of human cell lines, and very rarely in non-malignant cell lines (35, 36). The *in vitro* studies in which TGF β alone is capable of inducing EMT have either been performed in serum containing medium, which provides various growth factors commonly associated with wounding, including TGF β , FGF and EGF, and/or using high TGF β concentrations (2.5-10ng/ml) (36, 37). Expression profiling of genetic program underlying EMT of HaCat keratinocytes stimulated with TGF β to undergo EMT identified 80 EMT-related targets (27). Our expression profiling study distinguishes between genes regulated by TGF β without concomitant EMT in non-malignant HMEC and genes that are differentially expressed under conditions resulting in EMT.

We identified 10 genes specifically associated with EMT, five of which were not induced at all by TGF β alone. Transcriptional repression of E-cadherin, could be mediated by the transcription factor TCF8/ZEB1 (25). FGF2, whose transcript expression is significantly increased upon double treatment in this EMT model, has also been linked to E-cadherin repression and EMT (25, 26). Low E-cadherin immunoreactivity in breast cancer is associated with poor prognosis (38), whereas restoration of E-cadherin reverts the invasive phenotype of cancer cells (39). Irradiated HMEC redistribute E-cadherin from an insoluble to a soluble pool when cultured with TGF β , which is accompanied by a significant increase in N-cadherin. The cadherin switch from E- to N-cadherin frequently accompanies pronounced tissue reorganization in normal and pathological conditions (40). Studies on cancer cell lines indicate that N-cadherin is linked to a more malignant and invasive behavior (23). Consistent with this, double-treated HMEC were significantly more motile. Although double-treated HMT-3522 S1 cells were not invasive, double-treatment induced invasive behavior in HMT-3522 S2 cells, which are an EGF-independent strain of the HMT-3522 progression series.

Neither chronic TGF β signaling induced by irradiation nor supplementation with low TGF β concentrations was sufficient to induce the EMT phenotype in any of the three HMEC that were examined in our studies. EMT occurred only in irradiated HMEC in the presence of TGF β , even when TGF β was added 2 days post-IR. This suggested that a IR-induced event was a prerequisite for TGF β -mediated EMT that was sustained for 48h. IR initially induces a transient activation of Erk/MAPK *via* a ligand-independent mechanism (32). Although the involvement of Erk signaling in TGF β -induced EMT is controversial (41), many studies have shown a requirement for overexpression/mutational activation of elements of the Ras/Raf/Erk pathway for TGF β -mediated EMT (41-43). Furthermore, the importance of enhanced Erk activation in induction of EMT is also supported by studies in which Erk activity induced by Ras/TGF β (41), EGF/TGF β (31), HGF/ErB2 (44) and Akt (45), was found to be critically involved in EMT. Our data suggest a model in which IR-induced Erk/MAPK signaling is sustained by TGF β , is required for establishing and maintaining EMT, and is essential for the functional response, i.e. enhanced migration.

Cancer radiotherapy is primarily limited in many organs by the risk of developing fibrosis (46). Neilson and colleagues demonstrated EMT as a significant source of fibrosis in a kidney ligation model (47). An interesting implication from our study is that normal epithelia may undergo EMT in response to irradiation and TGF β , which could contribute to fibrosis following radiotherapy. If so, this would lend further credence to the potential application of TGF β inhibitors in radiotherapy.

Based on studies in mouse mammary gland, we proposed that the action of radiation as a carcinogen is augmented by its ability to modulate signaling from the microenvironment (48). Tumorigenesis is increased 4-fold when unirradiated preneoplastic mammary epithelial cells are transplanted to the mammary stroma of a host irradiated with 4 Gy (3). IR induces abundant TGF β activation (6). Our current and earlier studies (9) have shown that irradiated non-malignant HMEC undergo EMT only if they are exposed to additional TGF β , as might be derived from the stroma in intact tissues. If moderate radiation doses can prime preneoplastic cells to undergo EMT, it could accelerate cancer progression. Interestingly, a recent study shows that conventional renal cell carcinomas of the Ukrainian patients living in the radio-contaminated areas exhibited significantly higher levels of TGF β expression compared to similar tumors in populations in uncontaminated areas (49). These tumors were characterized by decreased or abnormal distribution of fibronectin, laminin, and E-cadherin/ β catenin, suggesting that chronic low level radiation exposure in humans might indeed shift tumors towards a mesenchymal phenotype. Nonetheless there is little evidence to support these events occurring in normal epithelia *in vivo* after moderate doses. Furthermore, additional studies from our laboratory indicate that TGF β is instrumental in mounting a DNA damage response (7, 20), and in inducing apoptosis of genomically unstable cells (Barcellos-Hoff, M.H. and Maxwell, C.A. unpublished observations). The complexity of radiation effects mediated by TGF β will require further study to determine whether it plays a proximal role in suppressing or promoting radiogenic carcinogenesis.

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Figure Legends

Figure 1

Loss of epithelial and gain of mesenchymal markers in irradiated HMEC in response to TGF β

(A) Immunolocalization of cytoskeleton-associated E-cadherin, β -catenin, ZO-1, F-actin (detected with phalloidin) (B) fibronectin, vimentin and N-cadherin in sham (untreated), IR, TGF β and double-treated (IR+TGF β) HMT3522 S1 cells. All images were acquired at 40x magnification. (C) Phase images of sham, IR, TGF β and IR+TGF β treated HMT3522 S1 (day 10), MCF10A (day 10) and 184v HMEC (day 7). The images were acquired at 10x magnification. All images in (A), (B) and (C) are representative of three independent experiments.

Figure 2

TGF β alters the cytoskeletal associated E-cadherin/ β -catenin complexes in irradiated HMEC

(A) Immunoblots and quantification of E-cadherin and β -catenin in total protein extracts from sham, IR, TGF β or IR+ TGF β HMT3522 S1 cultures. The graph represents quantification of E-cadherin protein abundance from nine independent experiments normalized to β -actin shown as mean \pm standard error. β -actin indicates equal protein loading. (B) Immunoblot of E-cadherin and β -catenin expression in E-cadherin immunoprecipitates from Triton soluble (S) and insoluble (I) protein extracts of HMT3522 S1 cells. (Student's t-test, p ** <0.0001)

Figure 3

TGF β induces an increase in motility and invasiveness in irradiated HMEC.

(A) Wound closure assay in sham, IR, TGF β and IR+ TGF β treated HMT3522 S1 cells. Representative images captured with a 10x objective at the time of wounding (0h) and 20h or 40h after wounding. All experiments were repeated at least 3 times, with similar results. (B) Quantification of transwell migration assay using HMT3522 S2 cells. Values represent mean \pm SE of total cell counts of each well from duplicate experiments. (C) Further propagation of MCF10A in 3D matrix without additional treatment. Sham, IR or TGF β -treated or double-treated MCF10A were grown to 80-90% confluency, trypsinized and embedded in MatrigelTM as previously described (9). Phase images of acini embedded in MatrigelTM were captured using 10x magnification. Acini were identified using the live wire tool from MIPAV and batch processed by in-house measurement routines (see material and method for details). Three separate experiments with multiple independent samples were analyzed. Averages of acini areas were plotted against their average shape factor for each sample. The shape factor is defined as $P^2/(4 \cdot \pi \cdot A)$ where P is the perimeter and A the area of the acinus. This value is equal to one for a perfect circle. A value greater than 1 is interpreted as the amount of deformation in comparison to a circle. Averages of acini areas were plotted against their average shape factor for each sample.

A p -value of $1.8E-5$ was obtained between sham and double treatment. All other groups could not be distinguished from sham. The circles on the scatterplot delimit the double-treated group compared to the other groups and indicates that double treatment leads to larger and more deformed acini. Statistical analysis of the 2D scatter plot between area and shape factor was performed by using multivariate analysis of variance (50).

Figure 4

Persistent effect of IR predisposes HMEC to TGF β -induced EMT

(A) Immunolocalization and quantification of SMAD in sham and IR-treated HMT3522 S1 cultures at day 6 post plating. The graph represents quantification of nuclear SMAD positive cells from one of three experiments (mean \pm SD; sham n=5464 cells, IR n=4876 cells). (B) Quantification of E-cadherin protein abundance from three independent experiments normalized to β -actin is shown as mean \pm SE. HMT3522S1 cells were grown for 10 days in the presence of 50% CM from control (sham-CM) or irradiated (IR-CM) cells in presence of TGF β neutralizing antibody or non specific IgG. (C) E-cadherin protein abundance in sham and IR-treated cells treated with TGF β neutralizing antibody (TGF β -NAb) or non specific IgG. Values are representative of 3 experiments normalized to β -actin shown as mean \pm SE. (Student's t-test, p * <0.01; p ** <0.001; p *** <0.0001)

Figure 5

Gene expression profiles of double-treated HMEC.

(A) Microarray transcript profiling for 10 EMT signature genes (FGF2, SERPINA1, IL1RL1, TCF8, SLC39A8, SFRP1, CRIP2, AKR1C2, CELSR2 and C9orf3) in sham, IR-treated, TGF β -treated and double-treated MCF10A cells 8 days post-IR. Biological replicates are shown. Although transcript levels of five of these ten genes (SERPINA1, SLC39A8, CRIP2, AKR1C2 and CELSR2) were significantly ($<p$, 0.05) higher in TGF β -treated samples than in sham samples, alterations changes were all below the 1.75-fold cut off used to define TGF β -responsive genes. The graph represents significance analysis of alterations in transcript expression levels (\pm SE) for the 10 EMT signature genes including the experiment shown in panel A and a replicate independent experiment (1 probe per gene except for FGF2 (2 probes) and SFRP1 (3 probes)), (B) Validation of microarray profiling for TCF8, FGF2 and CDH1 (E-cadherin) by real-time PCR. Increased TCF8 and FGF2 mRNA abundance is illustrated by a shift towards the left of the real time PCR curves in double-treated samples. CDH1 was decreased (right shifted curve) in TGF β -treated and double-treated samples. (Student's t-test, p * <0.05; p ** <0.001; p *** <0.0001).

Figure 6

Kinetics of MAPK signaling in HMEC as a function of IR and TGF β treatment.

(A) Erk and Mek activation in sham, IR, TGF β and IR+TGF β treated 184v HMEC. Sham, IR, TGF β and IR+TGF β treated cells were lysed 7 days (top panel) or 4-48h (bottom panel) post-IR, and immunoblotted with the indicated antibodies. Graphs represent densitometric analysis of immunoblots of phosphorylated Erk and/or Mek normalized to respective total protein. Error bars represent \pm S.E. (B) At 6 days post-IR 184v HMEC were treated with DMSO or U0126 (10 μ M). 8h after treatment cells were lysed, and immunoblotted with the indicated antibodies. (C) Sham, IR, TGF β and IR+TGF β treated MCF10A cells (6 days post plating) were cultured in DMSO or U0126 (10 μ M) for 30h, followed by staining for F-actin (detected with phalloidin) and E-cadherin. Representative images of only IR+TGF β treated cells are shown. E-cadherin and phalloidin images are representative of three and two independent experiments respectively. (D) Representative phase images of wound closure assay of sham, IR, TGF β and IR+TGF β in presence of DMSO or U0126 (10 μ M) at 0h and 14h. Images were acquired 14h after addition of DMSO or U0126. Phase images were captured using 4x objective. Three independent experiments were done and the graphs represent percentage of wound closure (\pm SD) at 14h after treatment from one such experiment.

Supplemental files

Two lists of up-regulated and down-regulated genes upon TGF β treatment are provided. As described in the Methods section, TGF β responsive genes are defined as genes for which at least three out of the four biological samples tested show a change of at least 1.75-fold compared to sham samples. In each list, genes are ranked from the most to the least responsive gene according to the averaged fold change in transcript level. The number of biological samples where the expression change is observed is indicated.

Figure 1

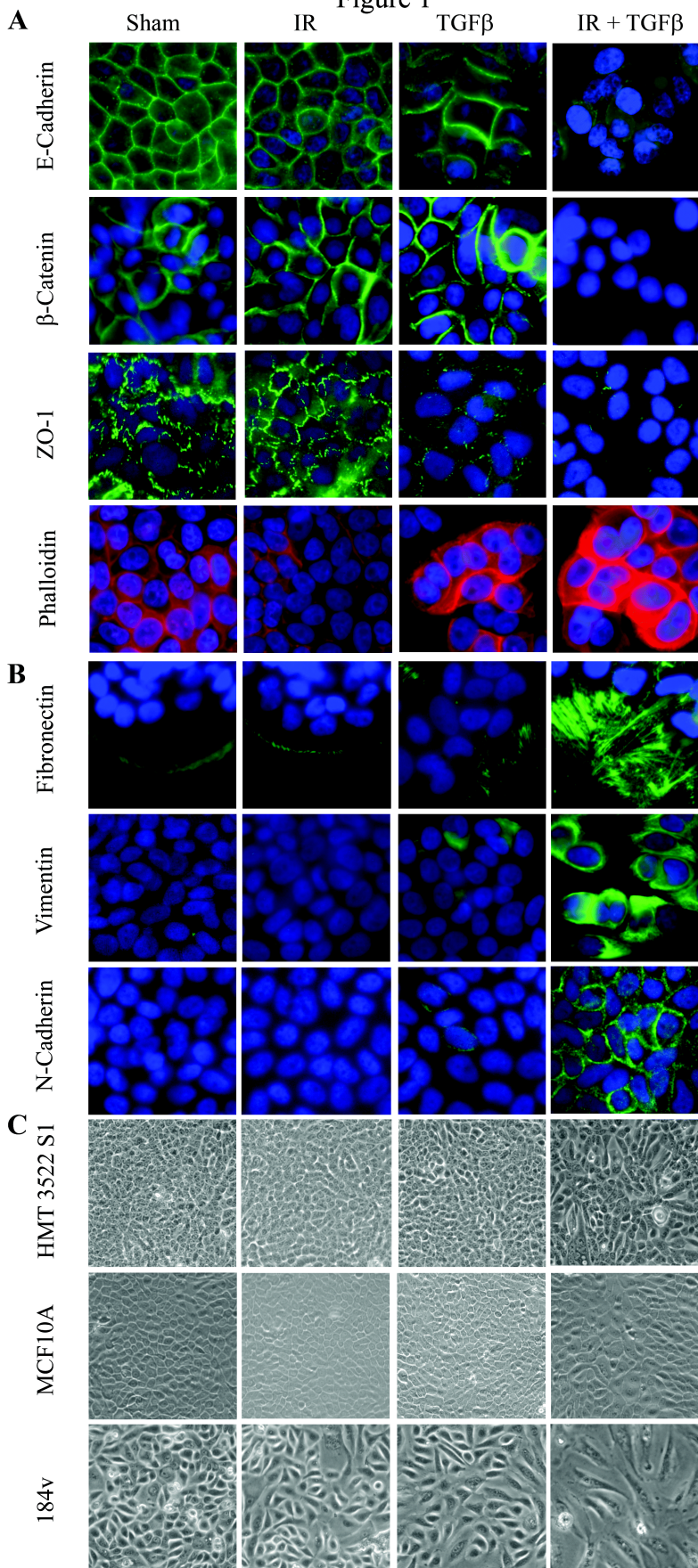
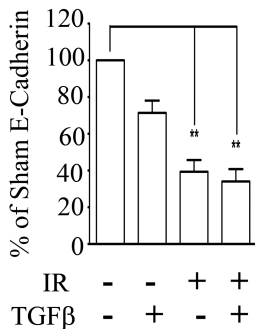
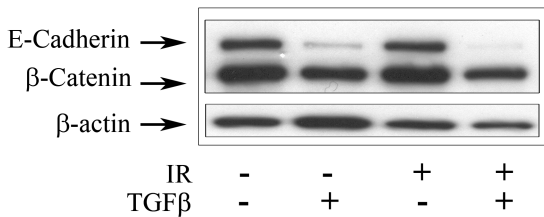


Figure 2

A



B

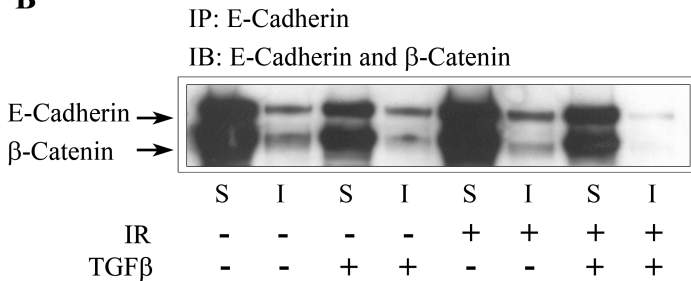
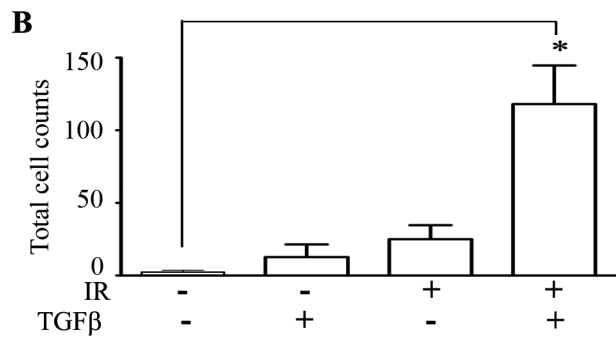
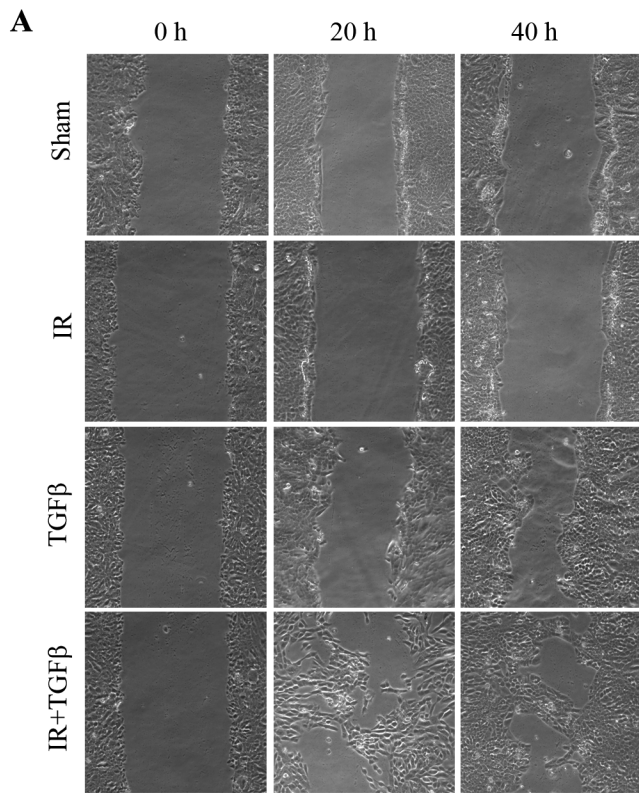


Figure 3



C

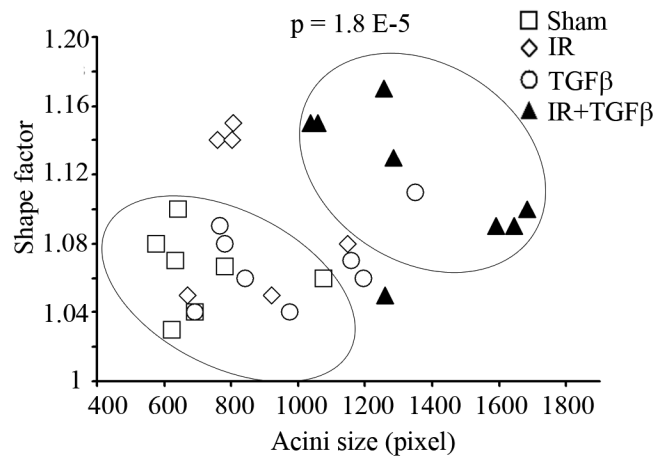
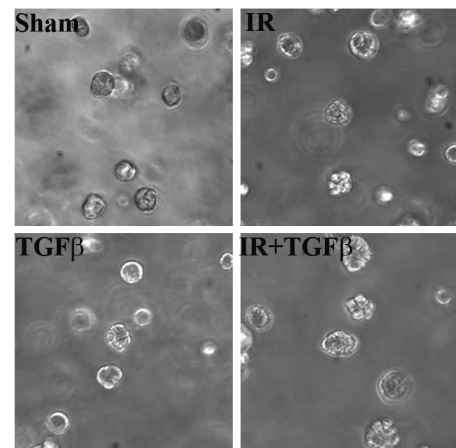
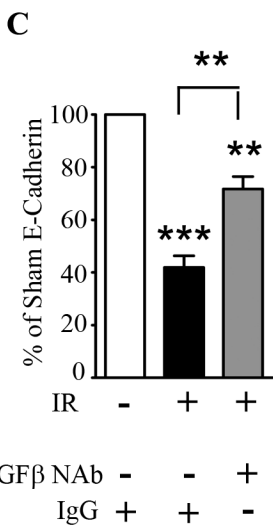
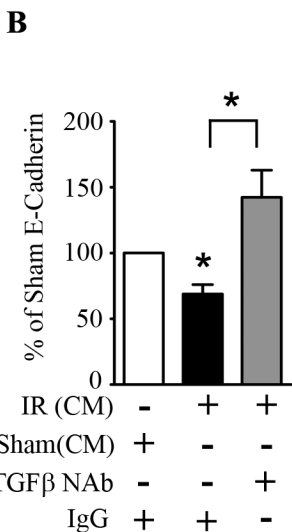
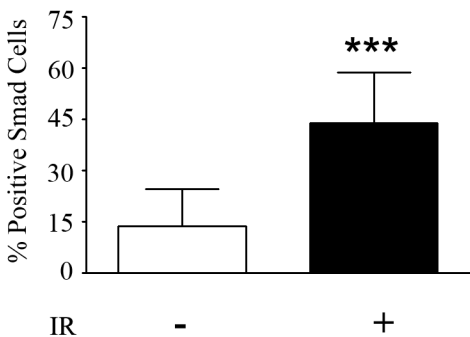
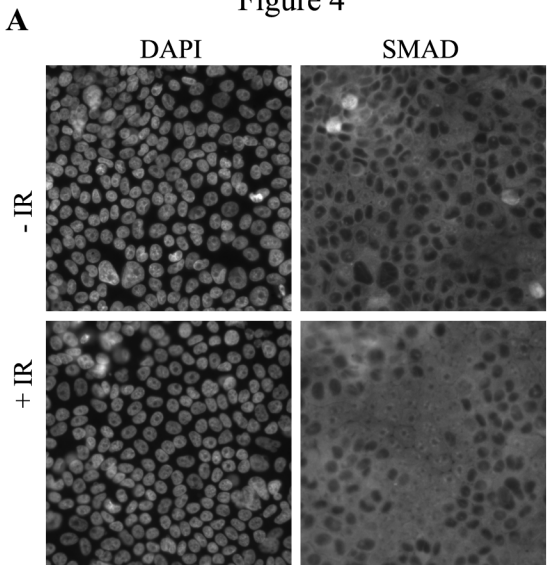
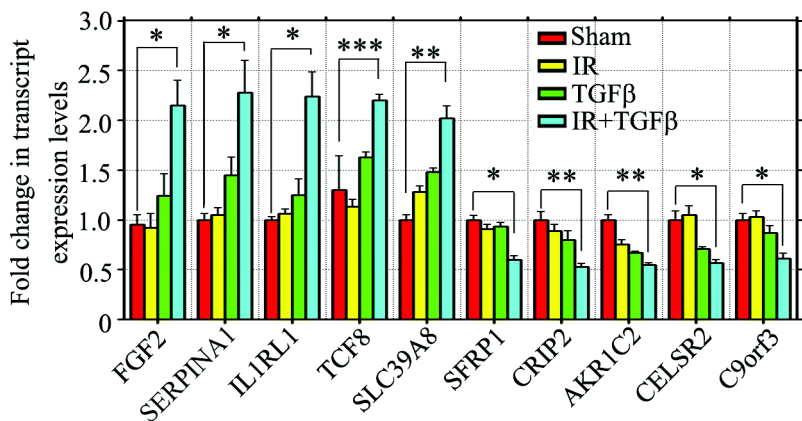
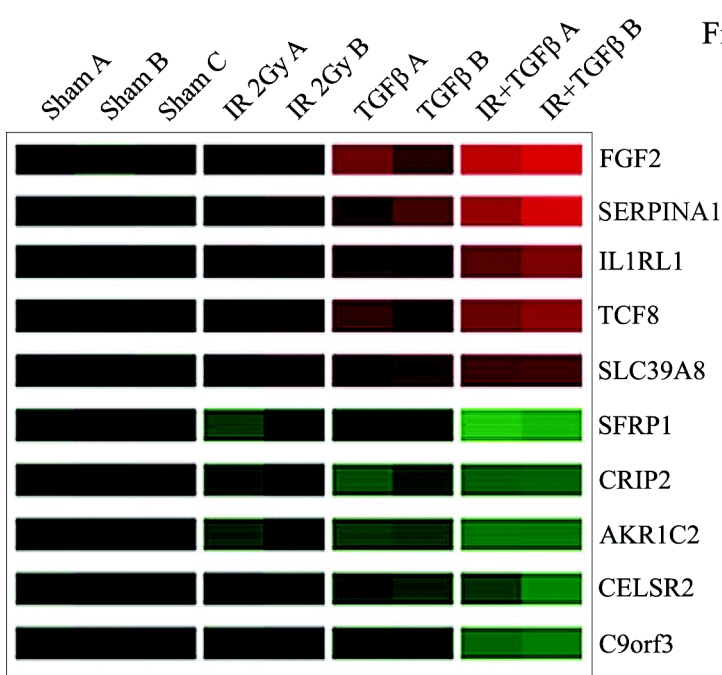


Figure 4



A



B

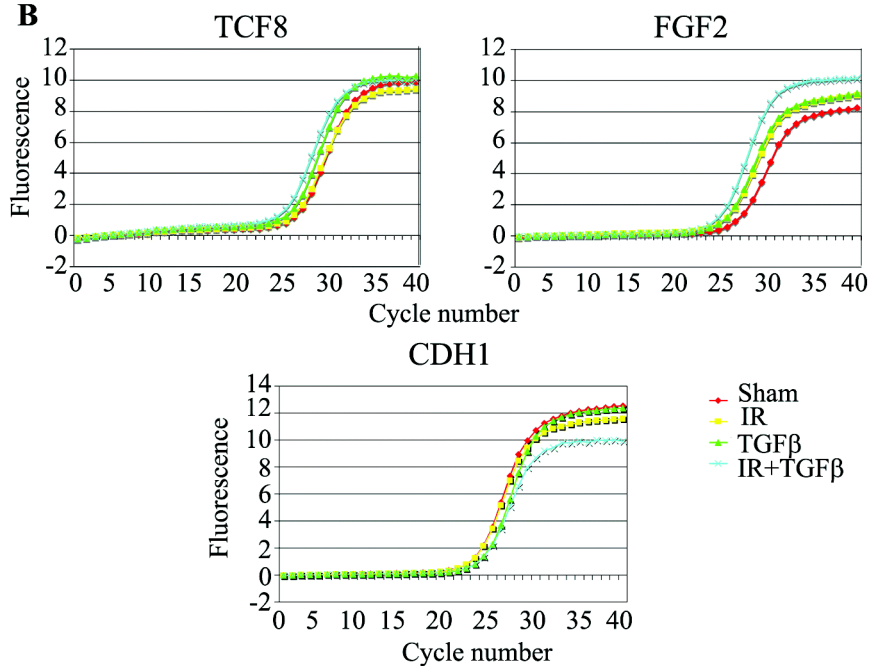


Figure 6

