Published in final edited form as:
J Cell Biochem. 2008 September 1; 105(1): 208–218. doi:10.1002/jcb.21814.

IFN-γ Regulation of Vacuolar pH, Cathepsin D Processing and Autophagy in Mammary Epithelial Cells

Zhila Khalkhali-Ellis[*,1,2], **Daniel E. Abbott**[1,3], **Caleb M. Bailey**[1], **William Goossens**[1], **Naira V. Margaryan**[1], **Stephen L. Gluck**[4], **Moshe Reuveni**[5], and **Mary J.C. Hendrix**[1,2]

[1]Children's Memorial Research Center, Northwestern University Feinberg School of Medicine, Chicago, Illinois, 60614-3394

[2]Robert H. Lurie Comprehensive Cancer Center, Northwestern University Feinberg School of Medicine, Chicago, Illinois, 60614-3394

[3]Department of Surgery, Northwestern University Feinberg School of Medicine, Chicago, Illinois, 60614-3394

[4]Department of Medicine, University of California, San Francisco, CA 94143-0114

[5]Department of Ornamental Horticulture, ARO Volcanic Center, 50250 Bet Dagan, Israel

Abstract

In this study we examined the ability of interferon-γ (IFN-γ) to regulate mammary epithelial cell growth and gene expression, with particular emphasis on two genes: *Maspin* (a member of serine protease inhibitor superfamily), and the lysosomal aspartyl endopeptidase *cathepsin D (CatD*). The protein products of these genes are critically involved in regulation of multitude of biological functions in different stages of mammary tissue development and remodeling. In addition, the expression of Maspin is down-regulated in primary breast cancer and is lost in metastatic disease, while CatD is excessively produced and aberrantly secreted by breast cancer cells.

We report that IFN-γ receptors are expressed in mammary epithelial cells, and receptor engagement by IFN-γ transduces the IFN-γ signal via Stat-1 resulting in decreased vacuolar pH. This change in vacuolar pH alters CatD protein processing and secretion concurrent with increased Maspin secretion. In addition, IFN-γ exerts a suppressive effect on cell growth and proliferation, and induces morphological changes in mammary epithelial cells.

Our studies also reveal that breast cancer cells, which are devoid of Maspin, are refractory to IFN-γ with respect to changes in vacuolar pH and CatD. However, Maspin transfection of breast cancer cells partially sensitizes the cells to IFN-γ's effect, thus providing new therapeutic implications.

Keywords

Maspin; IFN-γ; cathepsin D; mammary epithelial cell; breast cancer; vATPase; autophagy

The aspartyl endopeptidase Cathepsin D (CatD) is synthesized as a preproenzyme(von Figura & Hasilik, 1986) and subsequently undergoes post translational modifications to form the intermediate active 48 kDa (intermediate CatD) enzyme. Further cleavage into the two chain (34 and 14 kDa) mature enzyme occurs in the acidic lysosome (Kornfeld and Mellman, 1989; Hasilik and Neufeld, 1980). Under normal conditions ~10% of CatD

[*]Address Correspondence to: Zhila Khalkhali-Ellis, Ph.D., Children's Memorial Research Center, 2300 Children's Plaza, Box 222, Chicago, Illinois, 60614-3394, FAX: 773-755-6594, Email: zellis@childrensmemorial.org

escapes processing and is secreted (Dittmer et al., 1997), however aberrant and excessive secretion of CatD has been reported in many types of cancers (Rochefort et al.,1987; Garcia et al. 1990) and is associated with increased growth, invasive and metastatic attributes of the cancer phenotype.

Maspin, a member of clad 5 B serpins, is mostly a cytosolic protein but is also localized to the nucleus and membrane, and is secreted (Pemberton et al., 1997). In addition to its critical role in mammary gland development Maspin is involved in numerous biological processes including (but not limited to) apoptosis, cell motility, matrix remodeling, angiogenesis, regulation of cell phenotype and redox system (Shi et al., 2003; Sheng, 2004; Khalkhali-Ellis, 2006). The recent identification of Maspin's binding partners has been instrumental in deciphering new aspects of Maspin's molecular mechanisms (Blacque and Worrall, 2002; Bailey et al., 2005; Yin et al., 2005). Studies from our laboratory have indicated a close partnership between Maspin and CatD in the extracellular milieu (Khalkhali-Ellis and Hendrix, 2007). Specifically, secreted Maspin incorporated into the extracellular matrix inhibits CatD-mediated matrix degradation (Khalkhali-Ellis and Hendrix, 2007).

To identify the regulatory mechanism(s) governing CatD/Maspin expression (and interaction) and its significance in the mammary gland, we examined the ability of IFN-γ to regulate Maspin and CatD in normal mammary epithelial cells. IFN-γ exerts its pleiotropic function through binding to IFN receptor(s) (IFN-γ RI and II) (Jung et al., 1987). The interaction of two receptor subunits transduces the IFN-γ signal(Bach et al., 1996) by phosphorylating proteins such as Stat-1, which translocates to the nucleus and binds to cognate cis-acting IFN-γ activation sequences (GAS sites)(Darnell, 1998). Although downstream effects of IFN-γ such as regulation of cellular proliferation, differentiation (Lash et al., 2006; Haque et al., 2007; Hollenbaugh and Dutton, 2006) and apoptosis have been investigated in numerous systems, few studies address the significant effect of this cytokine on mammary tissue (Harvat, and Jetten 1996; Grunberg et al., 2000). In addition, the expression of IFN-γ receptors has not been established in normal mammary epithelial cells. Thus, the ensuing experimental approach was developed to examine the modulatory effect(s) of IFN-γ on mammary epithelial cells under normal and neoplastic conditions.

MATERIALS AND METHODS

Cell Culture

Normal human mammary epithelial cells HMEC (Cell Applications Inc, San Diego, CA) and HMEC1330 (Biowhittaker, Inc. Wakersville, MD) were maintained in defined mammary epithelial cell basal medium provided by the respective companies. As both cell lines gave comparable results in the experiments described in this manuscript, they are simply referred to as HMEpCs through out the text.

MCF-7, MDA-MB-231 breast cancer cells and their Maspin gene transfected counterparts (generated in our laboratory, Khalkhali-Ellis and Hendrix, 2007) were maintained in RPMI [containing 10% fetal calf serum (FCS) and gentamicin (50mg/l)]. Cultures were determined to be mycoplasma free using the GeneProbe rapid detection system.

Antibodies

Monoclonal antibody to IFN-γ RI was from Santa Cruz, while those against early endosome antigen-1 (EEA-1), lysosomal associated protein-1 (LAMP-1), Stat-1, phosphor-specific Stat-1 (pY701), Maspin and CatD were from BD Pharmingen. Polyclonal antibodies to IFN-γ R II and ZO-1 were from Santa Cruz, polyclonal goat antibody to CatD was from R&D Systems and antibodies against "E" (27kDa) and "a" (100kDa) subunits of v_0 ATPase were raised in the laboratories of Drs. Moshe Reuveni and Stephen L. Gluck respectively

(Reuveni et al, 2001, Sautin et al. 2005). Peroxidase labeled secondary antibodies for Western blot analysis were from GE Healthcare, and Alexa 488, 633 and 660 conjugated secondary antibodies for immunohistochemistry were from Molecular Probes.

IFN-γ Treatment of Normal Mammary Epithelial and Breast Cancer Cell Lines

IFN-γ (BD Pharmingen, 0.5-2.×10^8 Units/ml) treatment was performed on 60-75% confluent cultures at concentrations of IFN-γ ranging from 100-5000 pg/ml for 96 hrs In the case of breast carcinoma cell lines the media was exchanged with that containing 1% FCS and Mito+ serum free supplement prior to the treatment with INF-γ. Following the treatment regimen, conditioned media (CM) was collected and concentrated (Microcon Ultracell 10k MW cut off, Millipore Corporation, Bedford, MA). Cells were either taken in Trizol solution for mRNA extraction, or scraped and lysed in lysis buffer for subcellular fractionation.

For JAK 1 inhibitor studies, cells were treated with variable concentrations of the JAK 1 inhibitor Pyridone 6 [(p6) Calbiochem, 100-750 nmoles/l] for 2 hrs prior to the addition of IFN-γ. Cells treated with vehicle alone (DMSO) served as controls.

Real Time PCR

Total RNA was extracted using Trizol RNA isolation reagent (Life Technologies, Inc.). Total RNA (1 μg) was reverse transcribed using the Advantage PCR kit (Clontech). Real-time PCR was performed on a 7500 Real Time PCR System (Applied Biosystems, Foster City, CA) using TaqMan® gene expression human primer/probe sets for the following genes: Maspin (Hs00184728_m1), v_0ATPase (Hs00748673_s1), beclin1 (Hs00186838_m1) and cathepsin-D (Hs00157205_m1). cDNA (5 μl), 1.25 μl 20X Gene Expression Assay Mix, and 12.5 μl 2X TaqMan® Universal PCR Master Mix were mixed and 20μl was amplified with the following thermocycler protocol: 1 cycle at 50°C for 2 min; 1 cycle at 95°C for 10 min; and 40 cycles at 95°C for 15 seconds/60°C for 1 min. Target gene expression was normalized to the endogenous control gene GAPDH (GAPDH: 4333764F). Data were analy using Applied Biosystems Sequence Detection Software (Version 1.2.3). Each experiment was repeated twice and each sample was performed in triplicate.

Subcellular Fractionation

Cytosolic fractions were prepared as described previously (Khalkhali-Ellis and Hendrix, 2003). Briefly, control and IFN-γ treated cells were lysed in buffer A (10mM HEPES buffer pH 7.9 containing 10mM NaCl, 1mM DTT, 10% glycerol, 15mM $MgCl_2$, 0.2mM EDTA, 0.1%NP40, protease inhibitor cocktail, [Roche Applied Bioscience, Indianapolis, IN]), subjected to 3 freeze-thaw cycles and centrifuged (4500×g, 10 min) to yield a post-nuclear cytosolic fraction. The protein content of the fractions was determined using BCA reagent and 20μg total protein was used for Western blot analysis and CatD enzymatic activity assay.

CatD Activity Assay

The assay was performed in a 96-well black ELISA plate utilizing 20μg total protein in reaction buffer (0.1 M sodium acetate, 5mM DDT, 1mM EDTA, pH 5.5, final volume of 100μl) at room temperature and in the presence or absence of pepstatin (specific aspartyl protease inhibitor). Each sample was assayed in triplicate and the protease activity of CatD was measured by the addition (10μM) of the fluorogenic peptide substrate Mca-P-L-G-L-Dpa-A-R-NH_2 (R&D Systems, Inc) using the fluorescence plate reader FluoStar Optima (BMG Labteck GmbH, Durham, NC) with excitation and emission of 320nm and 405nm respectively.

Immunohistochemical Analysis of HMEpCs

The effect of IFN-γ on the expression of specific proteins was examined in HMEpCs grown either as a monolayer on glass coverslips or in transwell culture dishes (membrane pore: 4microns). Confluent cultures in transwell dishes polarized in 7-10 days as confirmed by staining a representative culture for Muc-1 which is reportedly an apical marker in mammary epithelial cells (Pimental et al. 1996,). HMEpCs under both culture conditions were treated with or without IFN-γ for 96 hrs. The CM was collected from top and bottom wells separately, concentrated 7x to determine apical and basolateral secretion of Maspin and CatD. The cells were fixed in 4% paraformaldehyde (or ice cold methanol) and permeablized in 0.1% Triton x-100/PBS. They were then treated with monoclonal antibodies against early endosome antigen-1 (EEA-1, an endosomal marker), lysosomal associated protein-1 (LAMP-1), polyclonal goat antibody to CatD, polyclonal rabbit antibodies against "E" (27kDa) and "a" (100kDa) subunits of v_0 ATPase, and ZO-1. The secondary antibodies were Alexa 488, 633 and 660 (Molecular Probes), or rhodamin conjugated.

Lysosomal pH Alteration and Its Effect on CatD Gene Expression and Protein Processing

The lysosomotropic agent chloroquine (CQ), and inhibitor of vATPase bafilomycin A1 (Baf, Sigma, St. Louis MO) were utilized to alter lysosomal pH. HMEpC cultures (60-75% confluent) were treated with CQ at 50-100μM and Baf at 50-100nM for time periods specified in the figure legends. The CM was collected and the cells were either taken in Trizol solution for mRNA extraction or lysed in lysis buffer for subcellular fractionation (described above). The vacuolar pH and its alteration in response to different treatment regimens was assessed using LysoSensor Yellow/Blue DND160 (Molecular Probe), an acidotropic agent which accumulates in acidic organelles as a result of protonation and emits yellow/blue light depending on the acidity of the particular organelle. LysoSensor was added at a final concentration of 2μM (2 min, 37°C), the cells were then washed with PBS and viewed either by fluorescence microscopy using a Dapi filter (280nm excitation, 460nm emission) or confocal microscopy.

Transmission Electron Microscopy (TEM)

Cells were harvested by trypsinization, peletted and fixed in 0.1 M sodium cacodylate-buffered pH 7.4, 2.5% glutaraldehyde and 2% paraformaldehyde solution for 2 hrs. The pellets were washed three times in 0.1 M sodium cacodylate buffer, pH 7.4, 7.5% sucrose, postfixed in 1% OsO_4 solution (2 h), dehydrated in an ethanol gradient (50-100% ethanol (10 min), and embedded in Araldite 502. Ultra thin sections were stained with uranyl acetate and lead citrate and examined in a JEOL 1200EX electron microscope (JEOL, USA, Inc. Peabody, MA).

RESULTS

IFN-γ Regulation of HMEpC Phenotype and Gene Expression

Western blot analysis of cytosolic fractions from control and IFN-γ treated HMEpCs confirmed the presence of IFN-γ receptor I and II and verified that receptor engagement by low concentrations (100-250pg/ml) of IFN-γ induced the expression of IFN-γ RI (~ 2 fold) without affecting receptor II, while higher IFN-γ concentrations reduced the level of both receptors (Fig.1A). Maximum induction was apparent by 10-24 hours, and longer incubation period reduced the receptor levels (data not shown).

INF-γ treatment of HMEpCs suppressed proliferation (~50% reduction in response to 250ng/ml IFN-γ, plateauing thereafter) and altered epithelial cell morphology (Fig.1B). The latter was further examined using polarized HMEpCs, which indicated the morphological changes were associated with disruption of cell polarity (at concentrations >1ng/ml),

internalization of the polarity-indicator apical marker Muc-1 and breach in cell-cell contacts confirmed by disrupted ZO-1 (zonula occluden, a tight junction scaffolding protein component) staining (Fig. 1C-I).

To determine whether the observed effect(s) of IFN-γ were associated with changes in CatD and Maspin expression, we utilized real time PCR analysis, which indicated no significant changes in the mRNA levels of either gene (data not shown). However, Western blot analysis of cytosolic extract and CM depicted a dramatic reduction of intermediate CatD (~48kDa) and secreted CatD with no apparent change in mature CatD (34kDa) compared to controls (Fig.2A&B). Although IFN-γ exerted minimal effect on cell associated Maspin (data not shown), it induced a concentration dependent increase in secreted Maspin which inversely correlated with the changes in secreted CatD (Fig. 2A). Other endosomal/ lysosomal markers such as early endosome antigen-1(EEA-1) and Lamp-1 were found minimally affected thus indicating that the observed effects were more specific to CatD (Fig. 2C). The changes in secreted Maspin and secreted CatD in response to IFN-γ were further examined under polarized conditions. This approach indicated that in polarized HMEpCs, secretion of Maspin and CatD is mostly at the apical surface. IFN-γ treatment, apart from its reciprocal effect on Maspin and CatD secretion, instigates a concentration dependent basolateral release of Maspin with limited effect on CatD secretion (Fig. 2D). Earlier studies indicated that vacuolar pH greatly affects processing of CatD, and lysosomotropic agents or inhibitors of vacuolar ATPase were shown to significantly alter CatD processing (Braulke et al., 1987). However, contrary to IFN-γ, these compounds prompted accumulation of intermediate CatD and reduced lysosomal mature CatD (Fig. 3E). Examination of the vacuolar pH using LysoSensor Yellow/Blue DND160 revealed decreased vacuolar pH (indicated by increased yellow fluorescence emission) in IFN-γ treated HMEpCs compared to control (Fig. 3A-C). In addition to lower pH, a considerable increase in vacuolar size and number was noted in IFN-γ treated HMEpCs (data not shown), with some vacuoles exhibiting intense yellow fluorescence emission after exposure to LysoSensor Yellow/Blue DND160 (arrow Fig. 3C).

Acidification of the lysosomal compartment is mostly achieved through the action of v H^+ ATPase (Stevens and Forgac, 1997), and changes in the assembly of the subunits and/or their recruitment to the membrane could alter the activity of this multi-unit enzyme (Kane, 1995, Gluck, 1996). Examination of mRNA from IFN-γ treated HMEpCs indicated a concentration dependent increase in the “e” subunit of the V_0 sector of vATPase compared to that of control (Fig. 3D). This was further confirmed by Western blot analysis of cytosolic fractions depicting differential expression of the 100kDa “a” subunit of the V_0 sector (Fig. 3E).

These observations prompted examination of the enzymatic activity of cell associated CatD which revealed ~3 fold enhanced activity of CatD in response to INF-γ (Fig.3F). Increased proteolytic activity of CatD, associated with changes in vacuolar size and pH, could reflect the requirement for increased intercellular proteolysis, a process intrinsic to autophagy which is known to occur during the mammary remodeling process (Motyl et al., 2006,Zarzyńska et al., 2007). To evaluate this possibility we examined the effect of INF-γ on the mRNA and protein expression of an autophagic marker beclin 1(Levine and Yuan, 2005), and observed a concentration dependent increase in both mRNA and protein (Fig. 4A-B). The presence of autophagosomes (some containing organelles) detected by TEM in HMEpCs in response to INF-γ further supported the real time PCR and Western blot analysis (Fig. 4C-E).

IFN-γ exerts its effects by binding to cognate receptors on target cells, thereby activating Stat-1 and leading to the transcription of a significant portion of IFN-γ induced genes

(Klampfer et al., 2007). Our studies also indicate that downstream effects of IFN-γ reported in this manuscript are associated with induced Stat-1 protein expression and its tyrosine 701 phosphorylation (Fig.5A). By utilizing the JAK 1 inhibitor Pyridone 6 (P6), we were able to demonstrate that P6 mitigated IFN-γ-induced Stat-1 phosphorylation, normalized CatD processing (Fig.5B), and inhibited vacuolar acidification and morphological changes in HMECs (Fig. 3D compared with 3B&C).

Effect of IFN-γ on MCF-7 and MDA-MB231 Breast Cancer Cell Lines

Although IFN-γ is utilized as a therapeutic agent for many types of cancers (DeVita Jr. and Rosenberg, 2001), the majority of breast cancer cell lines are found to be refractory to this cytokine [(Harvat and Jetten, 1996), Z-Khalkhali-Ellis, unpublished observation]. As a prelude to understanding the mechanism(s) of this nonconformity, we employed MCF-7 and MDA-MB-231 breast cancer cell lines and confirmed minimal expression of IFN-γ Rs in both cell lines (data not shown). These cell lines were refractory to INF-γ with respect to proliferation and changes in vacuolar pH (Fig.6). Indeed, application of LysoSensor revealed far fewer and more basic vacuolar networks in MCF-7 and MDA-MB-231 breast cancer cells compared to HMEpCs (Fig.6). In addition, IFN-γ exerted minimal effects on CatD processing in these cell lines (except a slight increase in MDA-MB-231 intermediate CatD at low IFN-γ concentrations) (Fig. 7A&C). These cancer cell lines are devoid of Maspin and IFN-γ treatment did not alter their Maspin expression. However, transfecting the *Maspin* gene rendered MCF-7 cells responsive to IFN-γ with respect to proliferation and CatD processing while it exerted minimal effect on the MDA-MB-231 cell line (Fig. 7B&D).

Both MCF-7 and their Maspin transfected counterpart formed polarized structures when cultured in transwell culture dishes (Fig. 7E). However, the disruption of tight junctions in response to INF-γ (as seen in HMEpCs) was only observed in Maspin transfected MCF-7 cells (Fig. 7F-I), mimicking the effects observed in HMEpCs. In addition, higher concentrations of INF-γ caused a modest increase in beclin 1 expression in Maspin transfected MCF-7. MDA-MB-231 and its Maspin transfected counterpart failed to polarize under the experimental conditions employed (Fig.8).

DISSCUSION

Although downstream effects of IFN-γ such as regulation of cellular proliferation, differentiation and apoptosis have been investigated in numerous systems, few studies address the significant effect of this cytokine on mammary epithelial cells under normal or neoplastic conditions (Harvat and Jetten, 1996; Grunberg et al., 2000). In addition, the presence of IFN-γ receptors on HMEpCs has not been previously reported. Our studies indicate that HMEpCs possess functional receptor I and II, and receptor engagement results in activating the JAK pathway, leading to phosphorylation of Stat-1 and downstream effects of IFN-γ such as reduced proliferation and loss of epithelial morphology. By utilizing polarized HMEpCs we observed disruption of cell polarity (at IFN-γ concentrations >1ng/ml) and breach in cell-cell contacts confirmed by disruption of the tight junction protein ZO-1. Disassembly of the tight junctions and abrogation of polarized structures are critical steps in normal tissue remodeling and pathogen-induced disruption of epithelial barriers (Ivanov et al., 2004). Such a process is observed in milk stasis (or mastitis) and could constitute a signal for initiation of mammary gland involution (Shamay et al., 2002).

Our studies indicate that IFN-γ decreases the pH of the vacuolar system in HMEpCs, thus altering secretion, processing and enzymatic activity of the aspartyl endopeptidase CatD. Increased proteolytic activity of CatD associated with changes in vacuolar size and pH could reflect the requirement for increased intercellular proteolysis, a process intrinsic to

autophagy which is known to occur during the mammary remodeling process (Motyl et al., 2006; Zarzyńska et al., 2007). Indeed, IFN-γ induced concentration dependent increases in mRNA and protein expression of an autophagic marker beclin 1(Levine and Yuan, 2005), concurrent with the appearance of autophagosomes (some containing organelles) was detected by TEM in HMEpCs. To our knowledge this is the first report of INF-γ induction of the autophagic process in mammary epithelial cells and could have important implications in mammary tissue homeostasis -- under normal and pathological conditions.

The effect of INF-γ on secretion of Maspin (and CatD) might reflect INF-γ induced changes in vacuolar biogenesis. Maspin is a member of clad 5B serpins and possesses a facultative secretion signal in its N-terminal region which determines it's bi-topological (inter- and extra-cellular) distribution (Pemberton et al., 1997). Reduced vacuolar pH could promote Maspin's Golgi to plasma membrane transport. This premise is indeed plausible given that inhibition of vATPase is shown to impede the intra-Golgi or Golgi to plasma membrane trafficking of other serpins (Yilla et al., 1993). It is noteworthy that in polarized HMEpCs, secretion of Maspin and CatD is mostly at the apical surface. IFN-γ treatment, apart from its reciprocal effect on Maspin and CatD secretion, instigates a concentration dependent basolateral release of Maspin with limited effect on apical release of CatD. This altered localized secretion could affect multiple biological functions of mammary tissue including differentiation, cell proliferation and extracellular matrix proteolysis. Studies (Laurent-Matha et al., 2005, Vetvicka et al., 1994) indicate that CatD acts as a mitogen via autocrine and paracrine effects, thus, reduced CatD secretion could be a rate limiting factor in cell proliferation. In addition, based on our previous findings (Khalkhali-Ellis and Hendrix 2007), secreted Maspin could incorporate into the extracellular matrix and regulate extracellular proteolysis and/or cell differentiation --processes fundamental to mammary gland remodeling. The actual occurrence of these events during mammary tissue remodeling is currently under investigation in our developmental mouse model.

Interestingly, the majority of breast cancer cell lines are refractory to INF-γ [(Harvat and Jetten, 1996), Z-Khalkhali-Ellis, unpublished observation]. This is further supported by our finding of minimal expression of IFN-γ Rs in breast cancer cell lines tested and the failure of IFN-γ to inhibit proliferation, vacuolar acidification and CatD processing in these cell lines. By utilizing LysoSensor we observed far fewer and more basic vacuolar networks in MCF-7 and MDA-MB-231 breast cancer cells compared to HMEpCs. This is in agreement with the reported observation that cancer cells have a diminished ability to acidify their lysosome/endosomes (Kokkonen et al., 2004). Of the two cancer cell lines tested, MCF-7 cells could form polarized structures, yet in contrast to HMEpCs these were impervious to the effect of IFN-γ. These cancer cell lines are devoid of Maspin, and IFN-γ treatment did not alter their Maspin expression. However, Maspin transfection of these cell lines rendered them more responsive to INF-γ. Specifically, polarized Maspin transfected MCF-7 displayed disrupted tight junctions (similar to those observed in HMEpCs) and a modest increase in beclin-1 when treated with 1ng/ml INF-γ. MDA-MB-231 and its Maspin transfected counterpart failed to polarize under the experimental conditions employed. The differential response of MCF-7 and MDA-MB-231 breast cancer cell lines to INF-γ is unclear and might be the consequence of their tumorigenic potential and differentiation status, which could also reflect the sensitivity of their Maspin transfected counterparts. Our findings define Maspin as a critical component of the INF-γ signaling pathway in mammary epithelial cells and illuminate (at least in part) why breast cancer cells are refractory to IFN-γ. The mechanism by which Maspin sensitizes these cancer cells to this cytokine is currently under investigation in our laboratory and has significant therapeutic implications.

In conclusion, this study provides evidence that IFN-γ receptors are expressed in mammary epithelial cells, and that receptor engagement by IFN-γ regulates vacuolar pH, CatD and

Maspin expression. This is achieved by increasing the vacuolar membrane associated V_0 sector of vATPase, leading to decreased vacuolar pH and increased CatD activity concomitant with the induction of autophagy, mostly through activation of JAK pathways. Novel aspects of IFN-γ's effects combined with the ability of Maspin to partially sensitize breast cancer cells to this cytokine might lead to identifying mechanisms which directly activate the antitumor effects of IFN-γ while avoiding its adverse systemic effects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors gratefully acknowledge Dr. Patricia M. Kane, Dept. of Biochemistry and Molecular Biology, SUNY Upstate Medical University, Syracuse, NY, for her advice and comments on the VATPase; Dr. Ming Lu, Department of Medicine, University of California, San Franciso, CA, for help with the pan "a" antibody of V_0 ATPase; and Drs. Paul Heidger and Khristofor Agassandian, Department of Anatomy and Cell Biology, The University of Iowa, Iowa City, IA, for counsel on TEM pictures and advice on the TEM protocol, respectively.

Contract grant sponsor: NIH/CA; Contract grant number: 75681

REFERENCES

Bach EA, Tanner JW, Marsters S, Ashkenazi A, Aguet M, Shaw AS, Schreiber RD. Ligand-induced assembly and activation of the gamma interferon receptor in intact cells. Mol. Cell. Biol. 1996; 16:3214–3221. [PubMed: 8649432]

Bailey CM, Khalkhali-Ellis Z, Kondo S, Margaryan NV, Seftor RE, Wheaton WW, Amir S, Pins MR, Schutte BC, Hendrix MJC. Mammary serine protease inhibitor (Maspin) binds directly to interferon regulatory factor 6: identification of a novel serpin partnership. J. Biol. Chem. 2005; 280:34210–34217. [PubMed: 16049006]

Blacque OE, Worrall DM. Evidence for a direct interaction between the tumor suppressor serpin, maspin, and types I and III collagen. J. Biol.Chem. 2002; 277:10783–1078. [PubMed: 11788595]

Braulke T, Geuze HJ, Slot JW, Hasilik A, von Figura K. On the effects of weak bases and monensin on sorting and processing of lysosomal enzymes in human cells. Eur. J. Cell Biol. 1987; 43:316–321. [PubMed: 3040410]

Darnell JE Jr. Studies on IFN-induced transcriptional activation uncover Jak-Stat pathway. J. Interferon Cytokine Res. 1998; 18:549–5454. [PubMed: 9726434]

De Vita, VTH., Jr; Rosenberg, S., editors. Cancer: Principles and Practice of Oncology. ed. 6th. Lippincot Williams and Wilkins; Hagerstown: 2001.

Dittmer F, Pohlmann R, von Figura K. The phosphorylation pattern of oligosaccharides in secreted procathepsin D is glycosylation site-specific and independent of the expression of mannose-6-phosphate receptors. J. Biol. Chem. 1997; 272:852–858. [PubMed: 8995373]

Garcia M, Derocq D, Pujol P, Rochefort H. Overexpression of transfected cathepsin D in transformed cells increases their malignant phenotype and metastatic potency. Oncogene. 1990; 5:1809–1814. [PubMed: 2284099]

Gluck SL, Underhill DM, Iyori M, Holliday LS, Kostrominova TY, Lee BS. Physiology and biochemistry of the kidney vacuolar H+-ATPase. Annu. Rev. Physiol. 1996; 58:427–445. [PubMed: 8815802]

Grunberg E, Eckert K, Karsten KU, Maurer HR. Effects of differentiation inducers on cell phenotypes of cultured nontransformed and immortalized mammary epithelial cells: a comparative immunocytochemical analysis. Tumour Biol. 2000; 21:211–223. [PubMed: 10867614]

Haque A, Das A, Hajiaghamohseni LM, Younge A, Banik NL, Ray SK. Induction of apoptosis and immune response by all-trans retinoic acid plus interferon-gamma in human malignant glioblastoma T98G and U87MG cells. Cancer Immunol. Immunother. 2007; 56:615–625. [PubMed: 16947022]

Harvat BL, Jetten AM. Gamma-interferon induces an irreversible growth arrest in mid-G1 in mammary epithelial cells which correlates with a block in hyperphosphorylation of retinoblastoma. Cell Growth Differ. 1996; 7:289–300. [PubMed: 8838859]

Hasilik A, Neufeld EF. Biosynthesis of lysosomal enzymes in fibroblasts. J. Biol. Chem. 1980; 255:4946–4950. [PubMed: 6989822]

Hollenbaugh JA, Dutton RW. IFN-gamma regulates donor CD8 T cell expansion, migration, and leads to apoptosis of cells of a solid tumor. J. Immunol. 2006; 177:3004–3011. [PubMed: 16920936]

Ivanov AI, McCall IC, Parkos CA, Nusrat A. Role for actin filament turnover and a myosin II motor in cytoskeleton-driven disassembly of the epithelial apical junctional complex. Mol. Biol. Cell. 2004; 15:2639–2651. [PubMed: 15047870]

Jung V, Rashidbaigi A, Jones C, Tischfield JA, Shows TB, Pestka S. Human chromosomes 6 and 21 are required for sensitivity to human interferonγ. Proc. Natl. Acad. Sci. USA. 1987; 84:4151–4155. [PubMed: 2954164]

Kane PM. Disassembly and reassembly of the yeast vacuolar ATPase in vivio. J Biol Chem. 1995; 270:17025–32. [PubMed: 7622524]

Khalkhali-Ellis Z, Hendrix MJC. Nitric Oxide regulation of maspin expression in normal mammary epithelial and breast cancer cells. Amer. J. Pathol. 2003; 162:1411–1417.23. [PubMed: 12707024]

Khalkhali-Ellis Z. Maspin: The new frontier. Clin. Cancer Res. 2006; 12:7279–7283. [PubMed: 17189399]

Khalkhali-Ellis Z, Hendrix MJC. Elucidating the function of secreted Maspin: inhibiting cathepsin D-mediated matrix degradation. Cancer Res. 2007; 67:3535–3539. [PubMed: 17440060]

Klampfer L, Huang J, Kaler P, Sasazuki T, Shirasawa S, Augenlicht L. Stat-1 independent inhibition of cyclooxygenase-2 expression by IFN-gamma; a common pathway for IFN-gamma mediated gene repression but not gene activation. Oncogene. 2007; 26:2071–2081. [PubMed: 17016440]

Kokkonen N, Rivinoja A, Kauppila A, Suokas M, Kellokumpu I, Kellokumpu S. Defective acidification of intracellular organelles results in aberrant secretion of cathepsin D in cancer cells. J. Biol. Chem. 2004; 279:39982–39988. [PubMed: 15258139]

Kornfeld S, Mellman I. The biogenesis of lysosomes. Annu. Rev.Cell Biol. 1989; 5:483–525. [PubMed: 2557062]

Lash GE, Otun HA, Innes BA, Kirkley M, De Oliveira L, Searle RF, Robson SC, Bulmer JN. Interferon-gamma inhibits extravillous trophoblast cell invasion by a mechanism that involves both changes in apoptosis and protease levels. FASEB J. 2006; 20:2512–2518. [PubMed: 17142800]

Laurent-Matha V, Maruani-Herrmann S, Prebois C, Beaujouin M, Glondu M, Noel A, Alvarez-Gonzalez ML, Blacher S, Coopman P, Baghdiguian S, Gilles C, Loncarek J, Freiss G, Vignon F, Liaudet-Coopman E. Catalytically inactive human cathepsin D triggers fibroblast invasive growth. J. Cell Biol. 2005; 168:489–499. [PubMed: 15668295]

Levine B, Yuan J. Autophagy in cell death: an innocent convict? J. Clin. Invest. 2005; 115:2679–2688. [PubMed: 16200202]

Motyl T, Gajkowska B, Zarzyńska J, Gajewska M, Lamparska-Przybysz M. Apoptosis and autophagy in mammary gland remodeling and breast cancer chemotherapy. J. Physiol. Pharmacol. 2006; 57(Suppl 7):17–32. [PubMed: 17228094]

Pemberton PA, Tipton AR, Pavloff N, Smith J, Erickson JR, Mouchabeck ZM, Kiefer MC. Maspin is an intracellular serpin that partitions into secretory vesicles and is present at the cell surface. J. Histochem. Cytochem. 1997; 45:1697–1706. [PubMed: 9389773]

Pimental RA, Julian J, Gendler SJ, Carson DD. Synthesis and intracellular trafficking of Muc-1 and mucins by polarized mouse uterine epithelial cells. J. Biol. Chem. 1996; 271:28128–28137. [PubMed: 8910427]

Reuveni M, Evenor D, Artzi B, Perl A, Erner Y. Decrease in vacuolar pH during petunia flower openingis reflected in the activity of tonoplast H^+ J. Plant. Physiol. 2001; 158:991–998.

Rochefort H, Capony F, Garcia M, Cavailles V, Freiss G, Chambon M, Morisset M, Vignon F. Estrogen-induced lysosomal proteases secreted by breast cancer cells: a role in carcinogenesis? J. Cell Biochem. 1987; 35:17–29. [PubMed: 3312245]

Sautin YY, Lu M, Gaugler A, Zhang L, Gluck SL. Phosphatidylinositol 3-Kinase-Mediated Effects of Glucose on Vacuolar H^+-ATPase Assembly, Translocation, and Acidification of intracellular compartments in renal epithelial cells. Mol. Cell. Biol. 2005; 25:575–589. [PubMed: 15632060]

Shamay A, Shapiro F, Mabjeesh SJ, Silanikove N. Casein-derived phosphopeptides disrupt tight junction integrity, and precipitously dry up milk secretion in goats. Life Sci. 2002; 70:2707–2719. [PubMed: 12269377]

Sheng S. The promise and challenge toward the clinical application of maspin in cancer. Front Biosci. 2004; 9:2733–2745. [PubMed: 15353310]

Shi HY, Zhang W, Liang R, Kittrell F, Templeton NS, Medina D, Zhang M. Modeling human breast cancer metastasis in mice: maspin as a paradigm. Histol. Histopathol. 2003; 18:201–206. [PubMed: 12507299]

Stevens TH, Forgac M. Structure, function and regulation of the vacuolar (H^+)-ATPase. Annu. Rev. Cell Dev. Biol. 1997; 13:779–808. [PubMed: 9442887]

Vetvicka V, Vektvickova J, Fusek M. Effect of human procathepsin D on proliferation of human cell lines. Cancer Lett. 1994; 79:131–135. [PubMed: 8019970]

von Figura K, Hasilik A. Lysosomal enzymes and their receptors. Annu. Rev. Biochem. 1986; 55:167–193. [PubMed: 2943218]

Yilla M, Tan A, Ito K, Miwa K, Ploegh HL. Involvement of the vacuolar H(+)-ATPases in the secretory pathway of HepG2 cells. J. Biol. Chem. 1993; 268:19092–19100. [PubMed: 8395529]

Yin S, Li X, Meng Y, Finley RL Jr. Sakr W, Yang H, Reddy N, Sheng S. Tumor-suppressive maspin regulates cell response to oxidative stress by direct interaction with glutathione S-transferase. J. Biol. Chem. 2005; 280:34985–34996. [PubMed: 16049007]

Zarzyńska J, Gajkowska B, Wojewódzka U, Dymnicki E, Motyl T. Apoptosis and autophagy in involuting bovine mammary gland is accompanied by up-regulation of TGF-beta1 and suppression of somatotropic pathway. Pol. J. Vet. Sci. 2007; 10:1–9. [PubMed: 17388018]

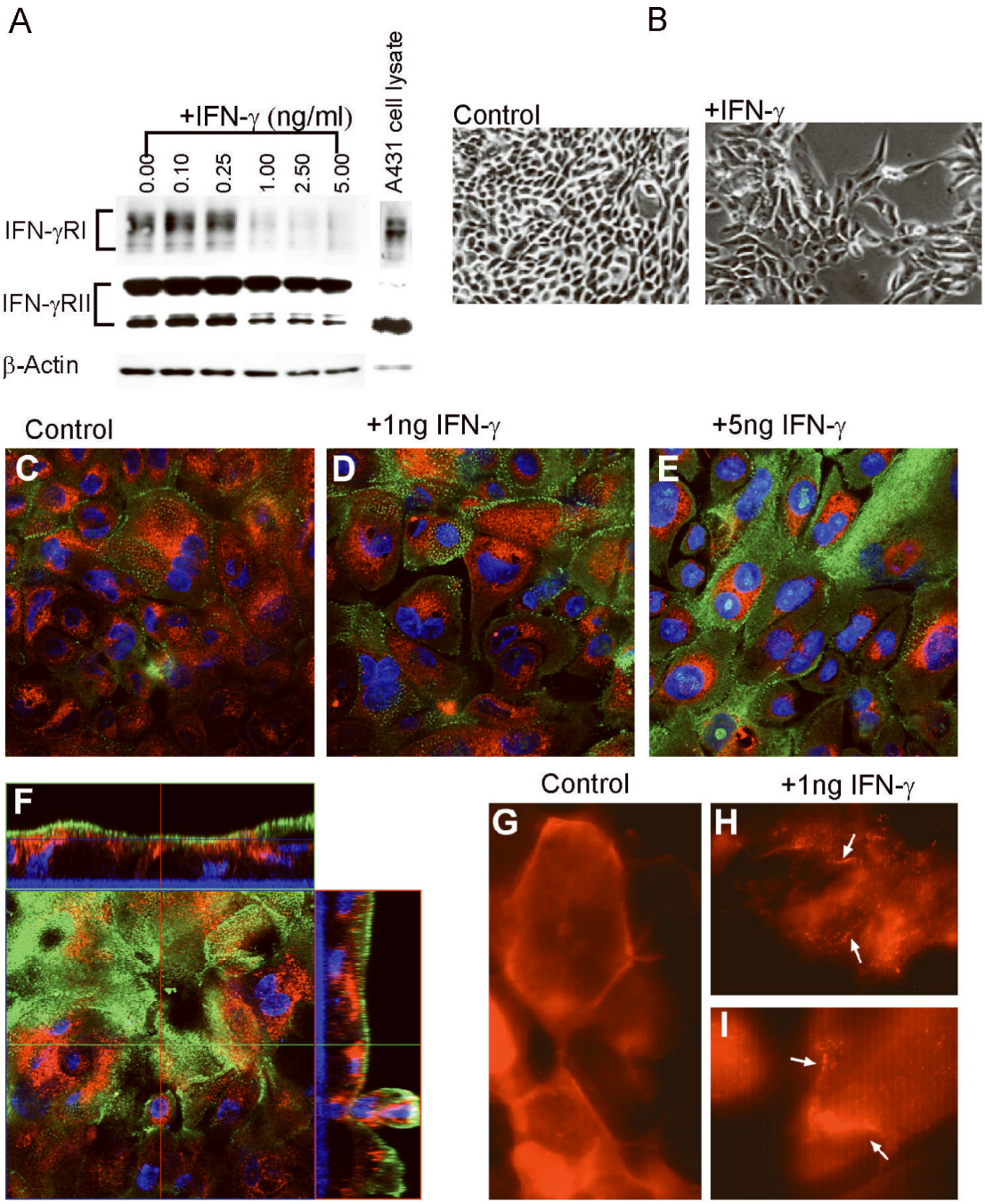

Fig. 1.

(A) Western blot analysis of post-nuclear cytosolic extract from control and IFN-γ treated HMEpCs demonstrates the presence of IFN-γ R I (multiple bands of~80-95 kDa), and RII (~38kDa major band and a smaller molecular mass of ~33-35kDa, most likely a cleavage product of the 38kDa form).

Changes in epithelial cell morphology in response to IFN-γ was assessed using monolayer cultures either on plastic (non-polarized) (B), or on transwell culture dishes (polarized) (C-I). Immunofluorescence staining of polarized HMEpCs with antibody to Muc-1 reveals the presence of Muc-1 (green fluorescence) at the apical surface determined by Z stacking of the confocal image (F). CatD staining (red) is included to indicate changes in CatD distribution

and intensity in control versus treated HMEpCs. Tight junctions are highlighted by ZO-1 staining (red fluorescence) of untreated HMEpCs (G), and their disruption (arrows) following IFN-γ treatment is depicted in (H-I). Original magnifications are 63x. C, F, and G depict control untreated and D, E, H, and I represent IFN-γ treated HMEpCs.

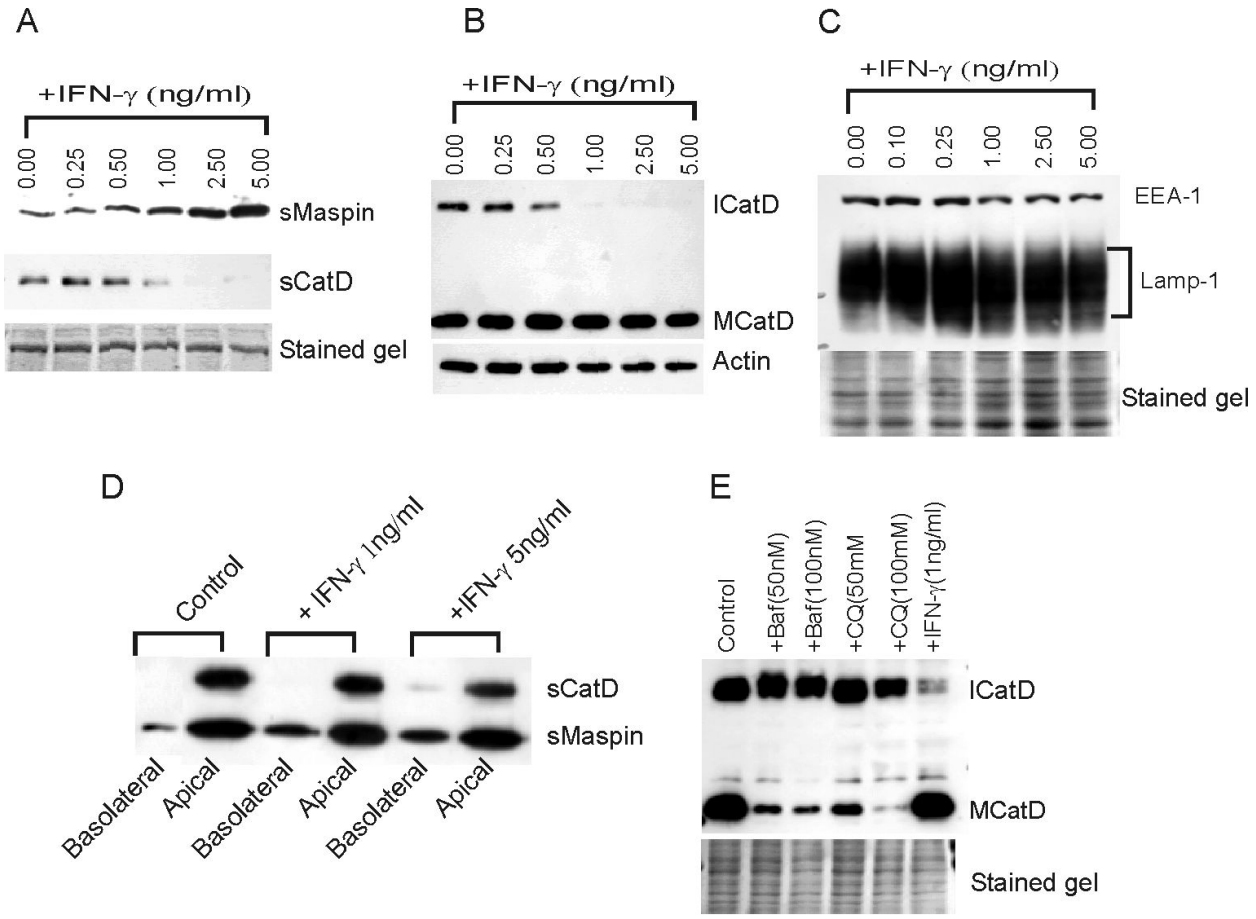

Fig. 2.

Western blot analysis of CM (A) and cytosolic extracts (B) from control and IFN-γ treated HMEpCs indicate concentration-dependent decreases in secreted CatD (sCatD) and intermediate CatD (ICatD) with minimal changes in 34kDa mature CatD (MCatD) (A&B). For comparative purpose, Western blot analysis of early endosomal antigen 1 (EEA1) and Lamp-1 are included (C) to indicate minimal effect on other endosomal/lysosomal enzymes. Note the inverse correlation between secreted Maspin (sMaspin) and secreted CatD (sCatD) in response to IFN-γ treatment (A). The secretion of Maspin and CatD in polarized HMEpCs are mostly at the apical surface and IFN-γ treatment instigates basolateral release of Maspin without affecting the CatD apical secretion (D).

Contrary to IFN-γ, chloroquine (CQ) and bafilomycin A (Baf) treatment of HMEpCs result in accumulation of ICatD and decreased MCatD (E). The SDS-PAGE was performed on a 10% (A, B & C) and 12.5% (D) resolving gel. For loading control either ß-actin or a picture of the stained post transfer gel is included.

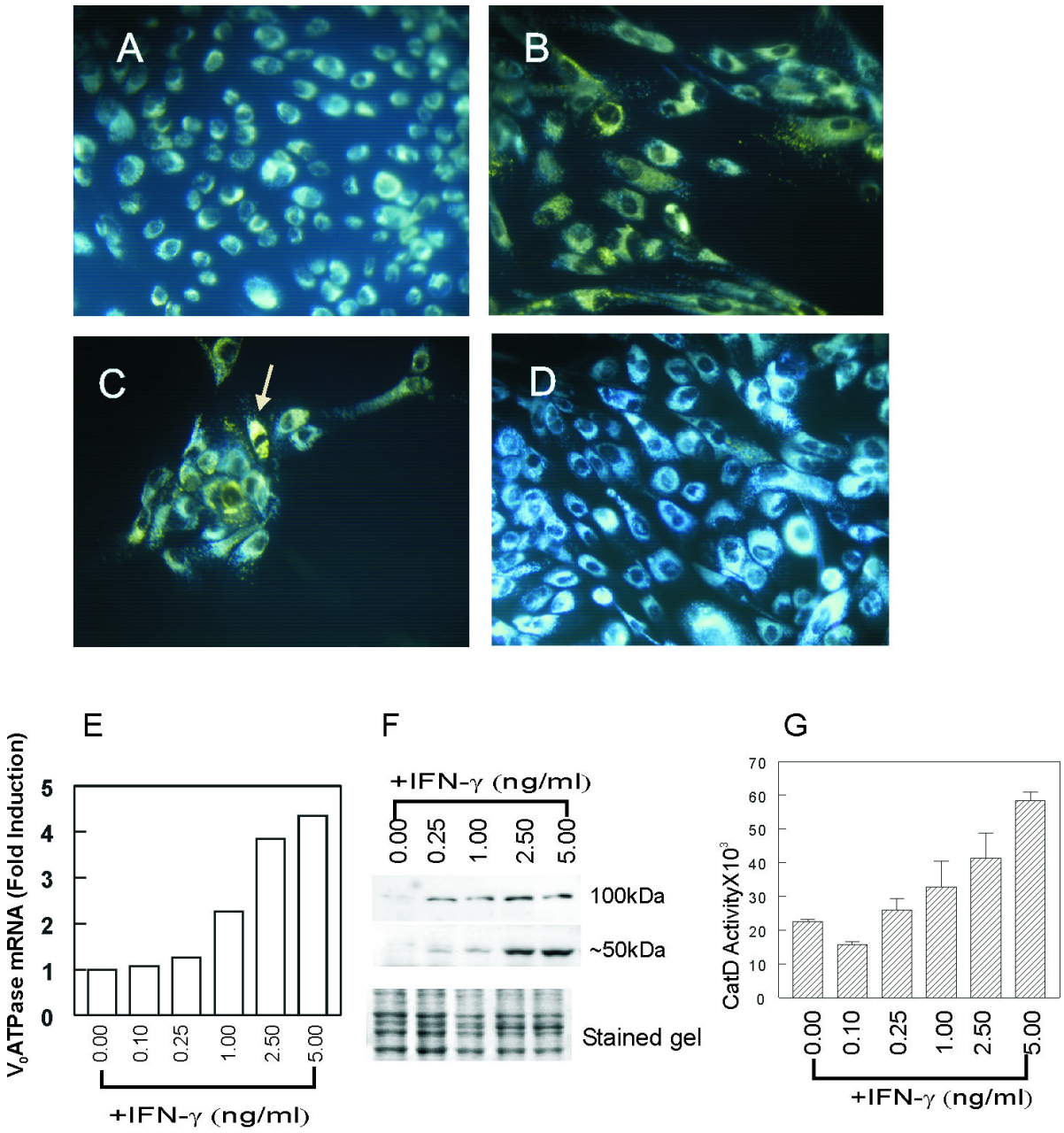

Fig.3.

Examination of vacuolar pH with LysoSensor prior to (A) and after IFN-γ treatment (B&C), 1 and 5 ng/ml IFN-γ, respectively indicates reduced pH resulting in increased yellow emission(arrow). The effect of IFN-γ on pH and CatD processing is abrogated using the JAK inhibitor p6 (D, magnification: 40x). PCR analysis of mRNA from the control and IFN-γ treated HMEpCs reveals a concentration dependent increase in the "E" subunit of v_0ATPase (E). This is confirmed by Western blot analysis of the cytosolic fraction of control and IFN-γ treated HMEpCs probed for the 100kDa subunit "a" of the V_0 sector of vATPase (F). The band corresponding to ~50kDa is most likely a degradation product of the 100 kDa subunit. Changes in cell associated CatD enzymatic activity in response to IFN-γ is depicted in (G). For loading control a picture of the stained post transfer gel is included in (F).

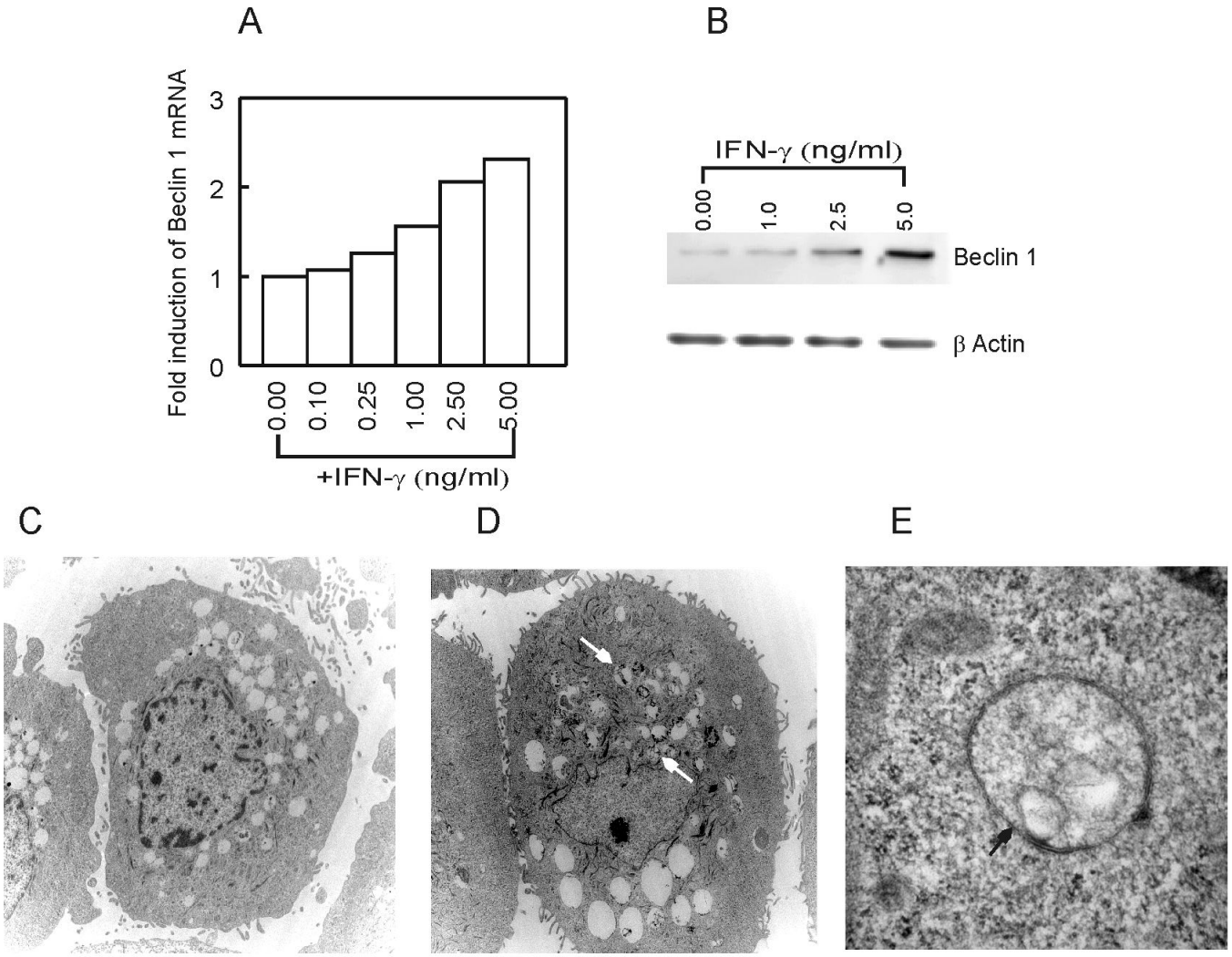

Fig. 4.

Concentration-dependent changes in beclin 1 mRNA (A) and protein expression (B) in HMEpCs in response to IFN-γ. TEM of control (C) and IFN-γ treated HMEpCs (D) indicate the presence of membrane bounded vacuoles (open arrow) in the HMEpCs treated with 1ng/ml IFN-γ. Autophagic vacuoles were identified by their characteristic double membrane (arrow, E). Note the numerous vacuoles (most probably secretory vacuoles) in both treated and control cells. Direct magnification is 10,000 (C&D) and 75,000 (E).

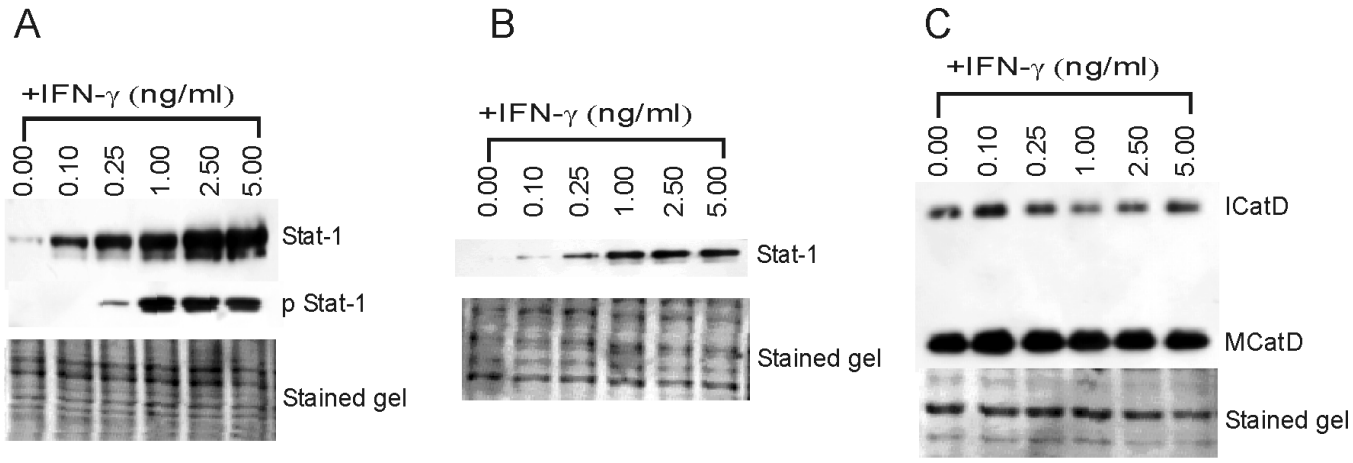

Fig.5.

Western blot analysis of cytosolic fractions from HMEpCs (prior to and after exposure to IFN-γ) depicts elevated Stat-1 expression and phosphorylation in response to increasing concentrations of IFN-γ (A). Utilizing the JAK inhibitor P6 mitigates the effect of IFN-γ on Stat-1 (B) and normalizes CatD processing (C). A 10% SDA-PAGE was used in these experiments and a picture of the stained gel is included to confirm equal loading. ICatD: intermediate CatD, MCatD: mature CatD.

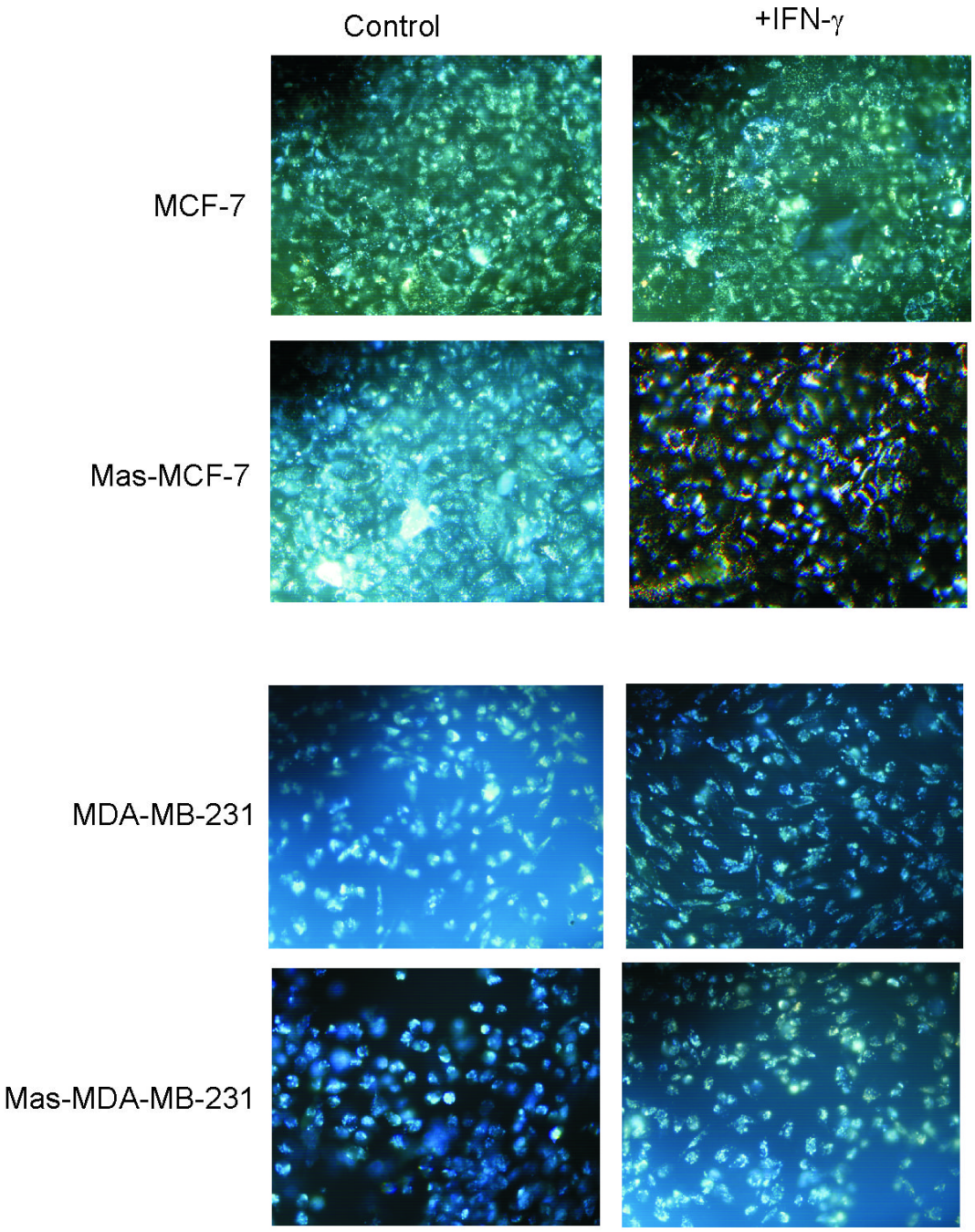

Fig. 6.

The vacuolar pH of MCF-7 and MDA-MB-231 breast cancer cell lines and their Maspin transfected counterparts prior to and after IFN-γ treatment was examined using LysoSensor Blue/Yellow DND160. Note the higher vacuolar pH in cancer cell lines compared to that of HMEpCs (depicted in Fig. 3 A-D of the manuscript).

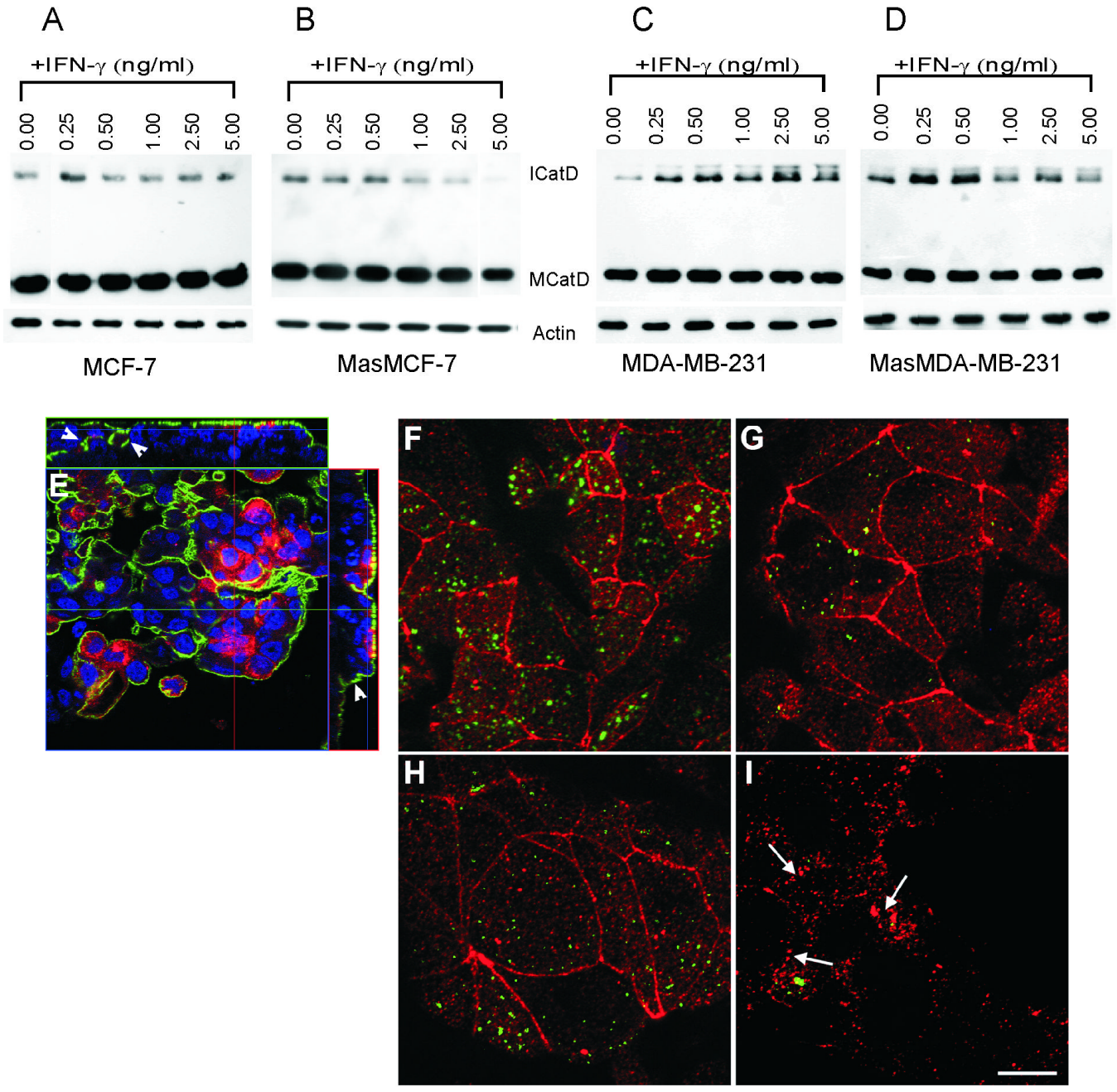

Fig.7.

Western blot analysis of post-nuclear cytosolic extracts from control and IFN-γ treated MCF-7 and MDA-MB-231 breast cancer cells indicate minimal changes in intermediate CatD (ICatD) (except a slight increase in MDA-MB-231 at low IFN-γ concentrations) in both cell lines (A&C). Maspin transfection of MCF-7 and MDA-MB-231 cells sensitizes them to IFN-γ effect (B&D). 10 % SDS-PAGE gels were used in these experiments. MCF-7 breast cancer cells form polarized structures determined by immunostaining with the polarity indicator Muc-1 (green) and Z stacking by confocal microscopy (E). The aggregates of MCF-7 formed in culture are also polarized (arrow heads). Red fluorescence depicts CatD and the nuclei are counterstained with Dapi. Examination of tight junction integrity with an antibody against ZO-1 indicates minimal effect of IFN-γ on tight junction assembly in

MCF-7 cells (F&G), while tight junction integrity was abrogated (arrows) in IFN-γ treated Maspin transfected MCF-7 (H&I). The green fluorescence (Alexa 488) depicts EEA-1 staining. Bar depicts 10μm and the original magnification is 63×10 with 2.5xscan zoom.

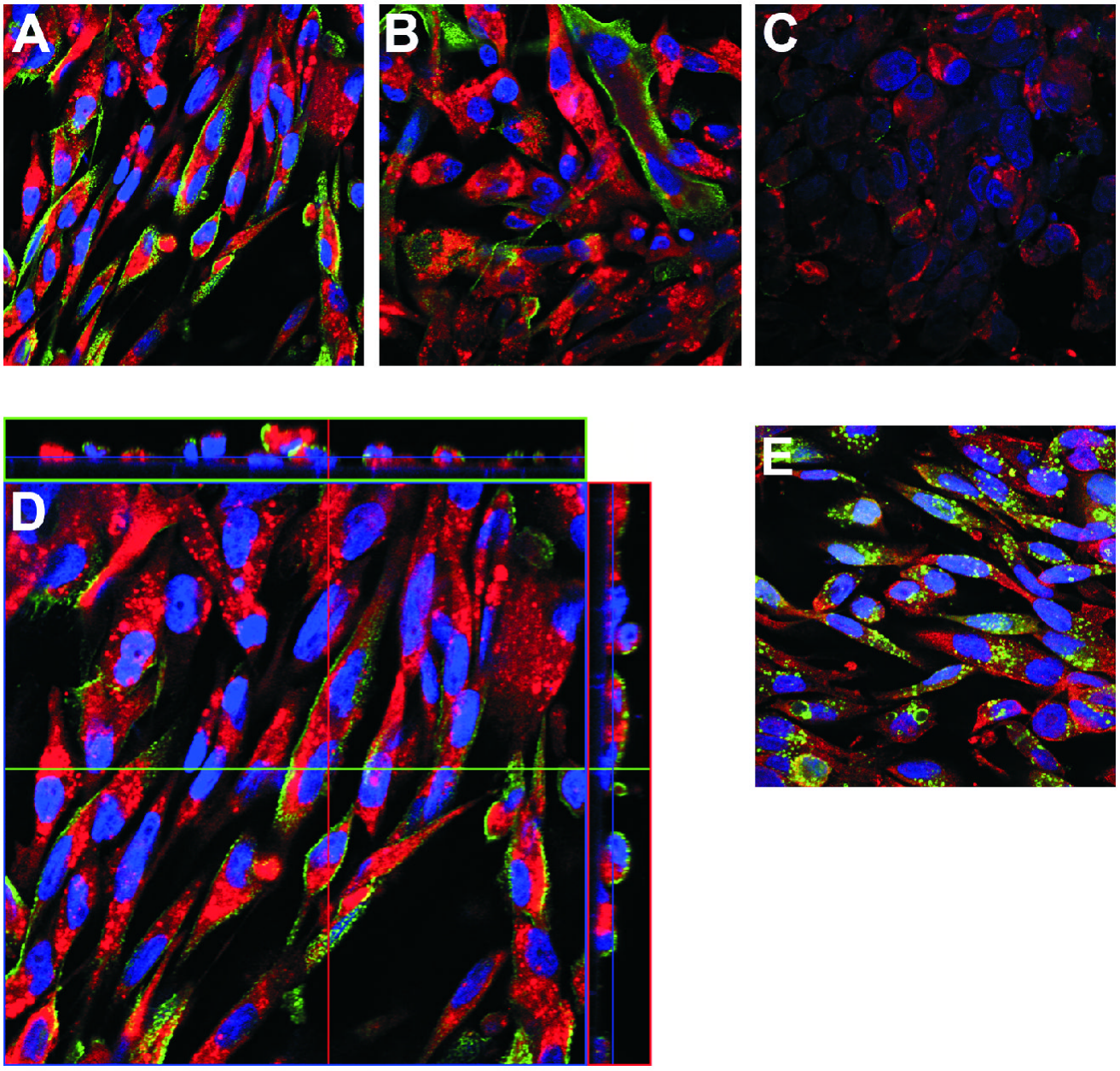

Fig.8.

MDA-MB-231 breast cancer cells fail to form polarized structures when cultured in transwell tissue culture dishes; Maspin transfection did not alter the response. Muc-1 (green fluorescence), CatD (red fluorescence, rhodamine) and the nucleus (blue, Dapi) immunostaining of control and IFN-γ treated (1 and 5ng/ml) cells are depicted in A-C, and Z stacking is shown in D. E represents control MDA-MB-231 immunostaining with anti-EEA-1 (green) and ZO-1 (red) to demonstrate the absence of tight junctions.