Short SULF1/SULF2 splice variants predominate in mammary tumours that are ideally suited to facilitate receptor tyrosine kinase mediated cell signalling

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ABSTRACT: The relative roles of SULF1 and SULF2 enzymes in tumour growth are controversial but short SULF1/SULF2 splice variants predominate in human mammary tumours despite their non-detectable levels in normal mammary tissue. Compared with the normal, the level of receptor tyrosine kinase (RTK) activity was markedly increased in triple positive mammary tumours during later stages of tumour progression showing increased p-EGFR, p-FGFR1 and p-cMet activity in triple positive but not in triple negative tumours. The abundance of catalytically inactive short SULF1/SULF2 variants permits high levels of HS sulfation and thus growth driving RTK cell signalling in primary mammary tumours. Also observed in this study, however, was increased N-sulfation detected by antibody 10E4 indicating that not only 6-O sulfation but also N-sulfation may contribute to increased RTK cell signalling in mammary tumours. The levels of such increases in not only SULF1/SULF2 but also in pEGFR, pFGFR1, p-cMet and Smad1/5/8 signalling were further enhanced following lymph node metastasis. The over-expression of Sulf1and Sulf2 variants in mammary tumour-derived MDA-MB231 and MCF7 cell lines by transfection further confirms Sulf1/Sulf2-mediated differential modulation of growth. The short variants of both Sulf1 and Sulf2 promoted FGF2-induced MDA-MB231 and MCF7 *in vitro* growth while full length Sulf1 inhibited growth supporting *in vivo* mammary tumour cell signalling patterns of growth. Since a number of mammary tumours become drug resistant to hormonal therapy, Sulf1/Sulf2 inhibition could be an alternative therapeutic approach to target such tumours by downregulating RTK-mediated cell signalling.

Introduction: Mammary tumours represent the most common cancer amongst women but like many other cancers it is molecularly heterogeneous and highly variable in its therapeutic response and metastatic properties (Engstrøm MJ et al. 2013). Molecular subtyping of this cancer provides some prognostic information regarding the most likely clinical outcomes. The most widely used markers for tissue biopsy characterisation include the positive or negative expression of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER-2), often described as triple positive or triple negative cancers. Many triple positive tumours are successfully treated over a number of years

although a proportion of them become drug resistant over time and thus effectively untreatable as are a large majority of the drug non-responsive triple negative cancers (Clarke R et al. 2015). It would thus help to discern the key drivers of breast tumour growth to enable targeting alternative signalling pathways to overcome drug resistant cell survival.

Cell signalling is one of the key drivers of tumour growth but most cancers activate not one but multiple signalling pathways. Targeting one specific cell signalling pathway often activates alternative pathways. While it is desirable to devise specific targets, the inhibition of multiple pathways may in some cases be more effective. For example, targeting co-receptors such as heparan sulfate proteoglycans (HSPGs) or their modifying enzymes may inhibit multiple cancer signalling pathways considering that the expression of HSPGs and their associated enzymes markedly change during tumour growth. Heparan sulfate (HS) chains of glycosaminoglycans (GAGs) in HSPGs are made up of repeating disaccharide units of glucuronic/iduronic acid and glucosamine that show further modifications of their properties generated by deacetylation, epimerization and sulfation at the N-, 3-O, and 6-O positions of glucosamine and the 2-O position of the iduronic acid residue. Changes in their HS sulfation patterns can exert differential functional effects on cell signalling and thus drive not only regulated normal development but also dysregulated tumour growth. Of particular interest amongst these changes is the 6-O sulfate group of the HS chains that has been shown to be associated with the regulation of a number of growth factor signalling pathways (Lamanna et al. 2007). The significance of 6-O sulfation in cell signalling has been further highlighted by the discovery of extracellular editing of HS sulfation by two cell surface sulfatases, Sulf1 (qSulf1) and Sulf2, which specifically hydrolyse glucosamine-6S groups of the HS chain (Dhoot et al. 2001; Morimoto-Tomita et al. 2002). Reduction in 6-O HS sulfation promotes certain cell signalling pathways such as Wnt and GDNF but inhibits others, for example tyrosine kinase receptor mediated FGF-2, HB-EGF and HGF pathways. Sulf1/Sulf2 regulation of 6-O HS sulfation thus has the potential to control cell growth and migration by regulating the activities of specific cell signaling pathways.

Both Sulf1 and Sulf2 have been shown to be upregulated in a number of cancer types (Bret et al. 2011; Gill et al. 2012; Gill et al. 2014; Nawroth et al. 2007; Lemjabbar-Alaoui et al. ; Phillips JJ et al. 2012) although some others have reported the down-regulation of Sulf1 in mammary tumours (Lai et al. 2006; Narita et al. 2007; Lai et al. 2008) since they describe Sulf1 as a tumour suppressor but Sulf2 as a tumour enhancer. Some such differences may relate to *in vivo* primary tumours versus *in vitro* cell line comparisons. While a number of tumour studies report the investigation of primary tumours many also invest a lot of effort in tumour derived cell lines, with each cell line representing a single patient considering that no two tumours are identical and even a single tumour demonstrates extensive spatial and temporal evolvement over time (Gill et al. 2014). The limited *in vitro* cell line analyses thus may not replicate the *in vivo* tumour growth patterns. The *in vitro* growth of cell lines also lacks *in vivo* competition for survival due to unsustainable active *in vivo* growth and stromal infiltration. The cell lines themselves can also evolve during different culture conditions and one has to bear in mind that normal mammary tissue does not express detectable levels of either Sulf1or Sulf2 proteins although low level of mRNA can be detected using multiple RT PCR amplification cycles (Morimoto-

Tomita et al. 2005). Cell lines nevertheless are a useful tool to investigate the role of different components in cell signalling and growth.

The additional problem of Sulf1/Sulf2 analysis of any pathological tissue and particularly the tumour tissues relates to the generation of multiple splice variants with different functional activities (Sahota and Dhoot 2009; Gill et al. 2011; Gill et al. 2012; Gill et al. 2014) that so far has received little attention. Lack of attention to splice variant diversity is even more misleading for those tumours in which the short variants of both Sulf1 and Sulf2 predominate during most stages of growth. The lack of catalytic activity in such shorter variants can completely change their cell signalling function. The present study therefore was undertaken to determine the nature of Sulf1 and Sulf2 variants in mammary tumours and to determine whether their ectopic expression alters tumour growth. This study demonstrates the abundance of shorter variants of both Sulf1 and Sulf2 in mammary tumours although low levels of full length isoforms were also detectable in such tissues. These tumours also demonstrate the up-regulation of RTK-mediated cell signalling pathways whose activities would be protected by shorter Sulf variants unlike the inhibitory effects of full length Sulf1 on RTK-mediated pathways. The *in vitro* ectopic expression of full length and Short Sulf1 and Sulf2 splice variants further supports their differential functions in mammary tumour-derived cell lines and their patterns of growth although most primary tumours as well as cell lines often express more than one variant in the same or adjacent *in vivo* stromal cells.

RESULTS:

Expression patterns of SULF1/SULF2 variants in mammary tumours: Neither SULF1 and nor SULF2 is detectable in normal mammary tissues using the immunohistochemical staining procedure (Figures 1 & 2). Low level SULF1 and or SULF2 expression is, however, detectable in some benign and hyperplastic samples (Figures 1 & 2). The onset of SULF1 expression and its persistence during all stages becomes increasingly clear in both triple positive and triple negative malignant tissues (Figures 1 & 2). SULF2 expression is also observed in all mammary tumours although the level of this enzyme was lower in triple positive compared with triple negative tumours (Figure 2). The shorter variants of both SULF1 and SULF2 enzymes were the most abundant isoforms in all mammary tumours. The levels of full length SULF2 and particularly SULF1 were low in all such tumours. While SULF1 expression appeared to be mainly in the epithelial or mesenchymal cells during all stages, the expression of SULF2 appeared relatively more abundant in the stromal tissue in both triple positive and triple negative tumours (Figure 2). The level of SULF1 in triple positive tissues was considerably higher than levels of SULF2 in such tumours while both SULF1 and SULF2 predominated in triple negative samples. The levels of full length SULF2 and particularly full length SULF1 in such tumours in comparison was much lower.

Cell signalling in mammary tumours: To determine if the low level expression of full length SULF1/SULF2 compared with much higher expression of their shorter variants impacted specific cell signalling pathways, we examined the activities of not only BMP and Hedgehog cell signalling but also markers for a number of tyrosine kinase receptor mediated cell signalling pathways during different stages of both triple positive and triple negative tumour growth. Compared with the normal,

immunohistochemical analysis demonstrated increased activation of BMP signalling investigated by the expression of p-Smad 1/5/8 staining as well as increased RTK staining for p-EGFR, p-FGFR1 and p-cMet (Figure 3). Semi-quantitative analysis of immunohistochemical staining using volocity software highlighted the onset of such signalling in all triple positive samples with particularly increased levels during stages II and III (Figure 4). While the level of p-Smad 1/5/8 and p-cMet in triple positive samples was low during stage I, the levels of p-EGFR and p-FGFR1 were high even during stage I. Unlike triple positive tumours, the levels of p-FGFR1 and particularly p-EGFR in contrast were low in triple negative tumours (Figure 4). While the level of BMP signalling also increased in triple negative tumours, the level of p-cMet in triple negative samples showed only a moderate increase. The AKT and ERK1/2 cascades are triggered by growth factors and cytokines acting through receptor tyrosine kinases. Investigation of such down-stream targets of RTK signalling pathways further indicated the active involvement of RTK cell signalling as a large majority of the tumours showed high levels of pERK and pAKT activity (Figure 5). A small proportion of the tumours, however, also showed greatly reduced level of pAKT and pERK activity not only in triple negative (Figure 5) but also in some triple positive tumours (Figure 5). Hedgehog signalling investigated by ptc1receptor expression did not show any specific changes in such tumours.

Increased SULF1/SULF2 expression and RTK-mediated cell signalling following mammary tumour metastasis to lymph node: We also examined if SULF1/SULF2 expression or cell signalling activities changed in a set of primary triple positive mammary tumours that had metastasised to lymph nodes when compared with the original tumour in its primary location. SULF1 as well as SULF2 expression was maintained and quantitatively enhanced following metastasis to lymph node although the level of SULF2 decreased during late stage metastasis (Figure 6). The nature of SULF1/SULF2 splice variant following metastasis did not change as the metastasised cells still predominantly expressed the shorter variants. . The semi-quantitative analysis of immunohistochemical staining using volocity software also showed significant increases in HSPG sulfation, p-EGFR, pFGFR1, p-cMet and BMP signalling analysed by p-Smad1/5/8 analysis during both earlier (stage II) and later (stage III) stages of metastasis (Figure 7).

The differential cell signalling roles of Sulf1/Sulf2 variants in some mammary tumour derived cell lines: To examine the role of Sulf1 and Sulf2 variants in tumour growth, triple negative MDA-MB231 and estrogen receptor positive MCF7 mammary tumour cell lines were individually transfected with full length and shorter Sulf1 and Sulf2 variants. MDA-MB231 cells express only a trace amount of full length Sulf1 under normal culture conditions but not any Sulf2 (Figure 8) since the DNA sequencing of a shorter band (indicated by an asterisk) in Sulf2 lane showed it to be a non-specific zinc finger protein. The RT PCR analysis of MCF7 cell line in contrast did not only confirm it to express high level of Sulf2 as reported by others (Uchimura et al. 2006) but also detected the expression of a short Sulf1 variant that has not been previously reported (Figure 8.b). The DNA sequencing of this fragment confirmed it to lack exons 6, 7 & 8. The Sulf1/Sulf2 gene transfection of these cell lines inducing not only mRNA but also protein expression was apparent in all cultures as is shown for MDA-MB231cells (Figure 8.c) that normally do not express any Sulf2.

The over-expression of full length Sulf1 (HS1) in MDA-MB231 cells inhibited its *in vitro* growth while the over-expression of the shorter Sulf1 (HS1-678) variant promoted growth. The over-expression of full length (HS2) as well as its shorter variant (HS2-678) in MDA-MB231cells promoted its growth although the level of increase with HS2 was higher than with HS2-678 transfection (Figure 8.d). The addition of FGF2 to such transfected cells promoted growth in EGFP and HS1-678 transfected cells and with only a marginal increase in HS2-678 transfected cells but reduced growth in HS2 transfected cells with a lower inhibition observed in HS1 transfected cells (Figure 8.d).

The over-expression of Sulf1 and Sulf2 variants in MCF7 cells promoted growth not only in HS1-678 and HS2-678 cells but also in HS2 transfected cells but with inhibition observed in HS1-transfected cells (Figure 8.e). The addition of FGF2 to such transfected cells demonstrated increased growth in EGFP and HS1-678 transfected cells but with no significant further growth but preservation in HS2-678 transfected cells (Figure 8.e).

The addition of cyclopamine to all MDA-MB231 transfected cells markedly inhibited *in vitro* growth while the addition of SHH ligand promoted growth in only the control EGFP transfected cells but inhibition in both full length and shorter Sulf1 and Sulf2 transfected cells (Figure 8.f). The addition of cyclopamine to all MCF7 transfected cells also markedly inhibited *in vitro* growth while the addition of SHH ligand promoted growth not only in EGFP but also in HS1 and HS2 transfected cells but demonstrated significant inhibition in HS1-678 and HS2-678 transfected cells (Figure 8.g).

DISCUSSION

The present study demonstrated SULF1 expression not only in triple positive but also in triple negative mammary tumours during all stages of tumour growth although the SULF1 level was much lower in benign tumours. This contrasts with little or no SULF1 expression in normal mammary tissue. The major SULF1 variant expressed in these tumours was the shorter variant lacking catalytically active exon 6 although low levels of full length enzyme were also detected in some tumours. Up-regulation of SULF2 was also observed in both triple positive and triple negative mammary tumours, with the level of SULF2 expression being higher in triple negative than triple positive. Although SULF2 was barely detectable in normal mammary tissue, significant levels of SULF2 were also observed in benign and hyperplastic mammary tissues. As was the case with SULF1, it was the shorter SULF2 splice variant lacking catalytically active exon 6 that predominated in such tumours although low but significant levels of full length SULF2 were also apparent in some samples. Alternative splicing of exons can alter protein function and thus increase the functional diversity of proteins. Such splicing could contribute to functional versatility of proteins to increase the diversity of interaction networks. Shorter variants could affect tumour growth by titrating out the activity of the full length inhibitory variant by competing for ligand or receptor binding. High levels of both SULF1/SULF2 in not only triple positive but also in triple negative mammary tumours, highlights the SULF1/SULF2 independence from estrogen signalling. This differs from estrogen receptor mediated EGFR-signalling that was barely detectable in triple negative tumours unlike its high activity in estrogen-positive tumours. This thus indicates

variation in the mechanism of SULF1 and SULF2 function in triple positive versus triple negative tumours. Short SULF1/SULF2 variants in triple positive tumours are ideally suited to promote 6-O sulfate requiring receptor tyrosine kinase signalling but it is not clear which signalling pathways are promoted by shorter variants in triple negative tumours. The level of BMP and Hedgehog signalling was higher in triple negative than triple positive tumours that may also be facilitated by short variants or the balance of full length and shorter variants in these tumours. Enhanced Wnt signalling and Notch signalling has also been reported to play a role in triple negative tumours (Jamdade VS et al. 2015; Koval A et al. 2016; King TD et al. 2012) although Wnt signalling is believed to be promoted by full length Sulf1 and Sulf2 genes.

The high levels of short SULF1/SULF2 variants observed during tumour growth also coincided with high levels of sulfation detected in triple positive as well as triple negative mammary tumours although 10E4 antibody has been reported to detect N-sulfation (David G et al. 1992) and not 6-O sulfation modulated by SULF1/SULF2. The low level sulfation in normal mammary tissue, however, is clearly not related to the presence of full length SULF1 or SULF2 but the differential activity of the intracellular sulfotransferases. It is thus not surprising to observe increased RTK-mediated cell signalling in mammary tumours, the activities of which would be enhanced by increased HS sulfation. For example, the level of EGFR and FGFR1 RTK activity was greatly increased in triple positive but not in triple negative samples except for some increase during stage II. The level of cMet RTK was similarly greatly increased in triple positive mammary tumours, particularly during stages II and III while the increase was lower but still significant in triple negative tumours. The presence of significant levels of short SULF1 and SULF2 variants would support such RTK-mediated cell signalling in mammary tumours. We nevertheless acknowledge that increased sulfation in mammary tumours observed by antibody 10E4 relates to N-sulfation and not 6,0 sulfation required for many RTK mediated cell signalling pathways. N-sulfation, however, has been reported to be important for certain cell signalling pathways. For example, defective N-sulfation of heparan sulfate proteoglycans has been shown to limit PDGF-BB binding and pericyte recruitment in vascular development (Abramsson A1 et al. 2007) although SULF1and SULF2 have also been shown to inhibit PDGF signalling by 6-O desulfation (Takashima Y1 et al. 2016). PDGF signalling is thus regulated by both N-sulfation and 6-O sulfation. N-sulfation or some factor requiring N-sulfation has also been shown to be important in the developing mammary gland (Bush KT1 et al. 2012).

Of all the RTK signalling pathways investigated in this study, the EGFR RTK activity demonstrated the clearest difference in triple positive versus triple negative tumours. For example, while triple positive mammary tumours demonstrated greatly increased EGFR RTK activity, triple negative tumours despite increased sulfation did not show any EGFR activity. Differences in EGFR cell signalling in triple positive versus triple negative samples could relate to lack of ER signalling in these tumours. EGFR signalling despite short Sulf1 and Sulf2 abundance regulating HSPG sulfation in triple negative tumours thus could not be promoted due to critical dependence of EGFR activity on ER signalling (Britton et al. 2006). Triple negative tumours nevertheless showed some pERK and pAKT downstream signalling due presumably to some activation of cMet RTK activity although even BMP signalling has been reported to increase activities of such enzymes in some cases (Ye L et al. 2009). For

example, the BMPs have been reported to affect RTK activity indirectly by promoting VEGF activity to enhance angiogenesis in many cancer tissues (Dai J et al. 2004; Deckers MM et al. 2002). A family of ligands that binds ErbB receptors (ErbB1/EGFR, ErbB2/Her-2/Neu, ErbB3 and ErbB4) plays an important role in breast cancer in which such receptors are over-expressed to promote tumour growth. When activated by ligand binding, this family of ErbB/EGFR receptor tyrosine kinases induces receptor dimerization, kinase activity and auto-phosphorylation to trigger downstream ERK1/2 and PI3K/AKT activation. Phosphorylation of such downstream signalling molecules enhances tumour cell survival and proliferation. The EGFR activity In human mammary cancers is thus implicated in regulating cell growth and survival through its differential activity via ER response. The constitutive activation of the kinase pathways, however, may bypass inhibition of EGFR/HER2 tyrosine kinases, and develop insensitivity to drugs targeting these receptors. This is compatible with reported observations (Britton et al. 2006) that estrogen receptor modulates EGFR/kinase signalling in some tamoxifen-resistant breast cancer cells.

To determine if SULF variant expression also correlated with some other cell signalling pathways, the immunocytochemical analysis also detected BMP signalling as revealed by downstream p-Smad 1,5,8 activation in both triple positive and triple negative tumours although some previous studies have shown BMP signalling restricted to only triple positive samples (Helms MW et al. 2005)**.** The precise function of BMP signalling in breast cancer, however, is still unclear since specific BMP ligands have been shown to promote as well as inhibit cancer cell growth and migration including metastasis (Alarmo EL 2010) although most such studies are restricted to mainly cell line analyses.

Breast cancer metastasis to lymph nodes showed further increase in not only BMP signalling but also RTK-mediated cell signalling including increased p-EGFR, p-FGFR1 and p-cMet activities. Increased RTK signalling following metastasis is also compatible with further increased expression of short SULF1 variant during both earlier and later stages and SULF2 variant increase only during earlier stage. RTK signalling is implicated in tumour invasion and metastasis but how such different signalling pathways are specifically used for tumour invasion is not clear. It is possible that increased RTK activation facilitated by increased sulfation de-stabilises E-cadherin adhesions to induce epithelial mesenchymal transformation (EMT) required for tumour cell metastasis to the lymph node or any other tissue (Qian X et al. 2004).

The involvement of Sulf1/Sulf2 splice variants in mammary tumour progression was further supported by our *in vitro* investigation of their over-expression in MCF7 and MDA-MB231 mammary tumour cell lines. The MCF7 cell line, however, unlike MDA-MB231 already expresses not only full length Sulf2 (Uchimura et al. 2006) but also a shorter variant of Sulf1 that we observed in the present study. Nevertheless, full length Sulf1 unlike the shorter Sulf1 variant inhibited MCF7 *in vitro* growth whereas over-expression of both full length and shorter Sulf2 promoted growth but with less clearer effects on FGF2 activity. It is also remarkable that the growth of MCF7 cell line was inhibited by full length Sulf1 considering that it already expresses high level of full length Sulf2 thus highlighting some inherent differences in Sulf1 and Sulf2 activities despite their high level of homology and similarity of function. It is possible that the expression of full length Sulf2 in MCF7 is sufficiently counteracted by the

presence of short Sulf1 variant so that full length Sulf1 can exert its inhibitory activity. Sulf1 over-expression in MDA-MB231 cell line that normally does not express any Sulfs with the exception of a trace amount of full length Sulf1 demonstrated clear differences in their effects on cellular growth as full length Sulf1 inhibited while short Sulf1 variant promoted *in vitro* growth. Unlike the full length Sulf1, over-expression of full length Sulf2 did not inhibit MDA-MB231 *in vitro* growth but instead promoted growth as did the shorter variant of Sulf2. The differential inhibitory effect of full length Sulf1 in comparison with growth enhancing effect of shorter Sulf1 was also apparent from their differential inhibitory and stimulatory effects on FGF signalling. While both short and full length Sulf2 promoted MDA-MB231 growth, the FGF2 activity was inhibited by full length but not the shorter Sulf2 variant. The mechanism of such full length Sulf1 and Sulf2 differences is not clear considering that both these enzymes have similar 6-O de-sulfation function and nor can it be explained by their differential cell surface signalling and ECM sequestering activities observed *in vivo* tissues. The SULF1 and SULF2 functional similarities observed in normal tissues thus not always hold true in tumour tissues due presumably to multiple mutations. Understanding the interactions between different components of cell signalling pathways could provide new avenues in devising novel therapeutic strategies for drug resistant mammary tumours. This study shows that both SULF1 and SULF2 are overexpressed in breast cancer in which they could promote tumour growth and metastasis. Targeting SULF1/SULF 2 may offer alternative strategies to develop novel cancer therapies. In conclusion, short SULF variants predominate in mammary tumours to stimulate growth and metastasis by protecting HS sulfation that enhances RTK-mediated cell signalling.

Materials and Methods

Tissue samples and immunocytochemical staining procedure: Human mammary tumour tissue arrays containing both normal and cancer tissue samples were obtained from US Biomax and included two or three cores from each patient sample. This analysis also included some other samples provided by Addenbroke hospital Cambridge as part of another study (Gill et al. 2011). The number of samples for each group were: normal: 11, hyperplasia: 6, benign: 6, triple positive invasive ductal carcinoma stage I: 35, stage II: 172, stage III: 75; triple negative invasive ductal carcinoma stage I: 6, stage II: 22, stage III: 12; number of triple positive invasive ductal carcinomas in primary location: 50 and following metastasis to lymph node: 50. The pattern of SULF1/SULF2 expression in such samples was examined using antibodies to these enzymes, the specificities of which have previously been described (Gill et al. 2011; Gill et al. 2012; Gill et al. 2010; Sahota and Dhoot 2009). Single or double immunofluorescence was used to stain paraffin sections of human tissues with different SULF1, SULF2 and some commercial antibodies (Gill et al. 2010; Gill et al. 2014) listed in table 1. The binding of mouse primary antibodies was detected using Alexa Fluor 488 fluorochrome-conjugated goat anti-mouse IgG diluted 1/400. Binding to total SULF1 (full length and all splice variants) by SULF1 antibody C (1/200) was visualised using goat anti-rabbit biotinylated IgG followed by streptavidin-conjugated Alexa Fluor 594. Sequential double immunofluorescence using two different rabbit antibodies included labelling exon specific antibodies with Alexa Fluor 488 followed by total SULF1 or SULF2

antibodies labelled with Alexa Fluor 594 (Figure 1.c). The double immunofluorescence procedure using two different rabbit antibodies sequentially always included staining with an exon specific antibody detected by Alexa Fluor 488 label first. The 2nd part of double immunofluorescence always included staining with an antibody to total SULF1 (antibody C) or total SULF2 (antibody D). To ensure no cross reaction with the two rabbit antibodies, the staining patterns were also ascertained with individual primary antibodies before their application in the double immunofluorescence procedure. Sections treated with pre-immune rabbit sera were similarly incubated with fluorochrome-labelled secondary antibodies as controls (not shown). All fluorochrome-labelled secondary antibodies were diluted 1/400. All appropriately diluted (table 1) primary antibody reactions were incubated overnight at 4 °C followed by secondary antibody incubations for 1 hour each at room temperature. Labelled tissue sections were mounted in fluorescent mounting medium (Sigma Aldrich) containing 2.5 µg/ml DAPI for nuclear visualisation and photographed using a Leica DM4000B fluorescent microscope. Quantification of the individual fluorescent images was carried out using Volocity software by measuring average pixel count/image.

Cell culture and transfection: Based on our splice variant analysis of canine and human Sulf1 and Sulf2 genes described previously (Gill et al. 2011; Gill et al. 2012; Gill et al. 2014), human Sulf1 and Sulf2 cDNA constructs cloned in pcDNA3 were transfected for over-expression in MCF7 and MDA-MB231 cell lines obtained from ATCC. Such cell lines purchased from ATCC were grown in RPMI (MCF7) or Dulbecco′s modified Eagle′s (DMEM) medium (MDA.MB231) with 10%FCS. Sulf1/Sulf2 variants were transfected with an EGFP expression vector while the control cells were transfected with EGFP/pcDNA3 alone using the Biorad TransFectin reagent. To enrich the mixed population of transfected cells, the normal growth medium was changed to DMEM/10%FCS with 800 µg/ml G418 following 48 hours of growth in the normal medium. The Sulf1/Sulf2 over-expression by transfection was confirmed by the RT PCR analysis. Such transfected cells were used for *in vitro* and *in vivo* assays following 2–6 weeks of growth in G418 containing medium. For *in vitro* proliferation assays, 10,000 cells/well were seeded in multiple 24-well plates and the trypsinised cells were counted after 5 days of culture. In addition to each sample consisting of a minimum of three replicates for each stage, each experiment was repeated three times.

RT PCR: To examine the expression of Sulf1 and Sulf2 splice variants in cell lines, total RNA was prepared from mammary tumour cell lines MCF7 and MDA-MB231 using Trizol. RNA was also prepared from these cell lines following their transfection with only EGFP (as control) and with full length or short Sulf1 or Sulf2 variant as described before (Gill et al. 2014). Equivalent amount of total RNA (1 µg) for each sample was reverse-transcribed into cDNA using SuperScript II reverse transcriptase (Invitrogen), using random primers (Invitrogen, Paisley, UK) for RT-PCR analysis. The PCR of cDNA was carried out using primers to exons 5 and 9 of the catalytic domain that we have shown to splice out exons 6–8 in different combinations in our earlier study (Gill et al. 2014; Zaman et al. 2016). The primers: 5'-CGAGGTTCAGAGGACGGATA-3' and 5'-GCCTCTCCACAGAATCATCC-3' were used to amplify 804-bp fragment of catalytic domain, nucleotides 83–886 bp of Sulf1. Primers, 5'-CAACTGTGTTCTCCCTGCTGGGT-3' & 5'-CTGGAGCATGTTGGTGAATTCC-3′ were used to amplify a region of Sulf2 catalytic

domain, nucleotides 38-843 (Zaman et al. 2016). PCR fragments following 40 amplification cycles separated in 2% agarose gels were cut out and purified to verify their identity by DNA sequencing (GATC Biotech). Primers 5′-CTATGAGCTGCCTGACGGTC-3′ and 5′-AGTTTCATGGATGCCACAGG-3′ were used to amplify 114bp β-actin (nucleotides 798–912 bp) to normalise the sample loading.

Statistical analysis: Statistical analysis was preformed using either a one-way ANOVA or a two-way ANOVA, where data depicting a P-value < 0.05 was considered statistically significant. To enable multiple comparisons a Tukey's test was used to determine significant variation between Sulf transfects to their eGFP controls or between full length Sulf isoforms and their shorter variants.

Conflict of interest: none

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Figure legends:

Figure 1: (a).: Pattern of SULF1 expression in tissue sections of normal, hyperplastic, benign and triple positive and triple negative ductal carcinomas during stages I, II and III examined by double immunofluorescence procedure. SULF1 is detected using antibody A against exon 6 (AlexaFluor488) and antibody C against total SULF1 (AlexaFluor594). The superimposed pictures show exon 6-containing SULF1 as yellow and exon-6 lacking SULF1 (total) as red. (b) shows semi-quantitative analysis of SULF1(yellow=full length; red=total) expression during different stages of triple positive and triple negative invasive ductal carcinoma growth in comparison with normal, hyperplastic and benign tumours. Quantification of the individual fluorescent images was carried out using Volocity software by measuring average pixel count, $^{**}p<0.001$, $^{*}p<0.01$. The number of samples for this analysis were: normal: 11, hyperplasia: 6, benign: 6, triple positive invasive ductal carcinoma stage I: 35, stage II: 172, stage III: 75; triple negative invasive ductal carcinoma stage I: 6, stage II: 22, stage III: 12. (c) highlights the location of Sulf1 and Sulf2 peptides used for antibody production (not to scale).

Figure 2: (a): Pattern of SULF2 expression in tissue sections of normal, hyperplastic, benign and triple positive and triple negative ductal carcinomas during stages I, II and III examined by double immunofluorescence procedure. SULF2 is detected using antibody N against exon 6 (AlexaFluor488) and antibody D against total SULF2 (AlexaFluor594). The superimposed pictures show exon 6-containing SULF2 as yellow and exon-6 lacking SULF2 (total) as red. (b) shows semi-quantitative analysis of SULF2 (yellow=full length; red=total) expression during different stages of triple positive and triple negative invasive ductal carcinoma growth in comparison with normal, hyperplastic and benign tumours. Quantification of the individual fluorescent images was carried out using Volocity software by measuring average pixel count, $^{**}p<0.001$, $^{*}p<0.01$. The number of samples for this analysis were:

normal: 11, hyperplasia: 6, benign: 6, triple positive invasive ductal carcinoma stage I: 35, stage II: 172, stage III: 75; triple negative invasive ductal carcinoma stage I: 6, stage II: 22, stage III: 12.

Figure 3: Level of N-sulfation (using antibody 10E4) and growth factor signalling activities (using antibodies to phosphorylated EGFR, FGFR1, cMet and Smad,1,5,8) in normal mammary tissue and during different stages (I, II, III) of triple positive invasive ductal mammary tumours examined by immunofluorescence (AlexaFluor488) superimposed with blue DAPI nuclear stain.

Figure 4: Semi-quantitative analysis of HS N-sulfation (a) and activities of different cell signalling pathways (b-f: as indicated on the side of each set of histograms) in normal, hyperplastic and benign tumour tissues and during different stages of growth of both triple positive and triple negative invasive ductal carcinomas. The individual fluorescent images were used for pixel count to quantify relative levels of expression using Volocity software, $^{**}p<0.001$, $^{*}p<0.01$. The number of samples for this analysis were: normal: 11, hyperplasia: 6, benign: 6, triple positive invasive ductal carcinoma stage I: 35, stage II: 172, stage III: 75; triple negative invasive ductal carcinoma stage I: 6, stage II: 22, stage III: 12.

Figure 5: Patterns of pERK and pAKT signalling activities revealed by red immunohistochemical stain (superimposed with blue nuclear DAPI stain) in normal and three different stage III triple positive and triple negative patient samples of invasive ductal carcinoma. The staining patterns demonstrate regional variation as well as variation in the level of their activities in individual patient biopsies.

Figure 6: (a) & (b): Patterns of SULF1 and SULF2 expression in triple positive invasive ductal carcinomas in their primary location in mammary tumour and the same tumour following metastasis to lymph node. SULF1 is detected using antibody A against exon 6 (AlexaFluor488) and antibody C against total SULF1 (AlexaFluor594). The superimposed pictures show exon 6-containing SULF1 as yellow and total SULF1 as red. SULF2 is detected using antibody N against exon 6 (AlexaFluor488) and antibody D against total SULF2 (AlexaFluor594). The superimposed pictures show exon 6-containing SULF2 as yellow and total SULF2 as red. Also shown in this figure is semi-quantitative analysis of changes in SULF1 (a1) and SULF2 (b1), (yellow=full length; red=total).

Figure 7: (a): Pattern of pEGFR1 expression in triple positive invasive ductal carcinoma in its primary location in mammary tumour and the same tumour following metastasis to lymph node, with p-EGFR staining appearing green with blue DAPI nuclear counterstain. (b-g) show semi-quantitative analysis of changes in N-sulfation (b) and different growth factor signalling activities (pEGFR, pFGFR1, p-cMet, ptc1, Smad1,5,8) as indicated in triple positive invasive ductal carcinoma stages II and III in primary mammary tumour location and following metastasis to lymph node, $^{**}p<0.001$, $^{*}p<0.01$. The number of samples for this analysis were: triple positive invasive ductal carcinomas in primary location: 50 and following metastasis to lymph node: 50.

Figure 8: (a) & (b): Pattern of Sulf1/Sulf2 expression and the over-expression of full length Sulf1 (HS1), full length Sulf2 (HS2) and shorter Sulf1 (HS1-678) and shorter

Sulf2 (HS2-678) by transfection in MDA-MB231 (a) and MCF7 (b) cell lines examined by RT PCR analysis as are also the cells transfected with only EGFP. (c): In addition to RT PCR, transfected cells were also examined by EGFP expression and Sulf2 expression by immunofluorescence, i=MDA-MB231 cells transfected with HS2 showing eGFP expression, ii= MDA-MB231 showing Sulf2 protein expression by immunofluorescence, iii= untransfected MDA-MB231 cells showing lack of Sulf2 protein expression by immunofluorescence. The effect of individual Sulf1 and Sulf2 variant over-expression was examined on growth of such cells over 5 days in both the presence and absence of 50ng/ml FGF2 in MDA-MB231 cells (d) and MCF7 (e) cells. Also examined was the effect of Sulf1 and Sulf2 variant over-expression on Hedgehog signalling stimulation using SHH (10ng/ml) and inhibition by Cyclopamine (10μM) on the growth of MDA-MB231 (f) and MCF7 (g) cells following 5 days *in vitro* growth, error bars: means±SD of samples, $^{**}p<0.001$, $^{*}p<0.01$. Each proliferation assay included a minimum of three replicates, and each experiment was repeated three times.

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Figure 1

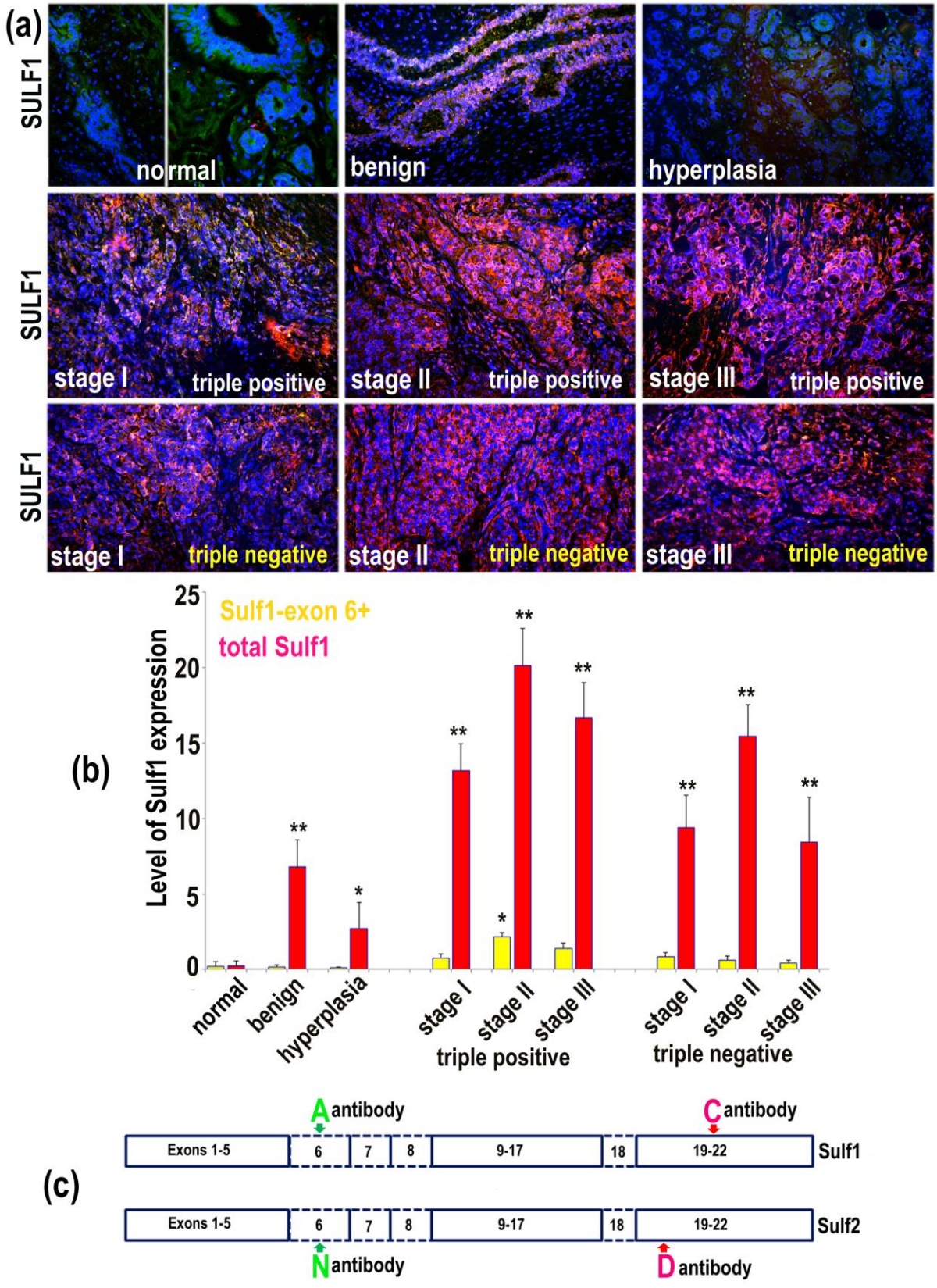
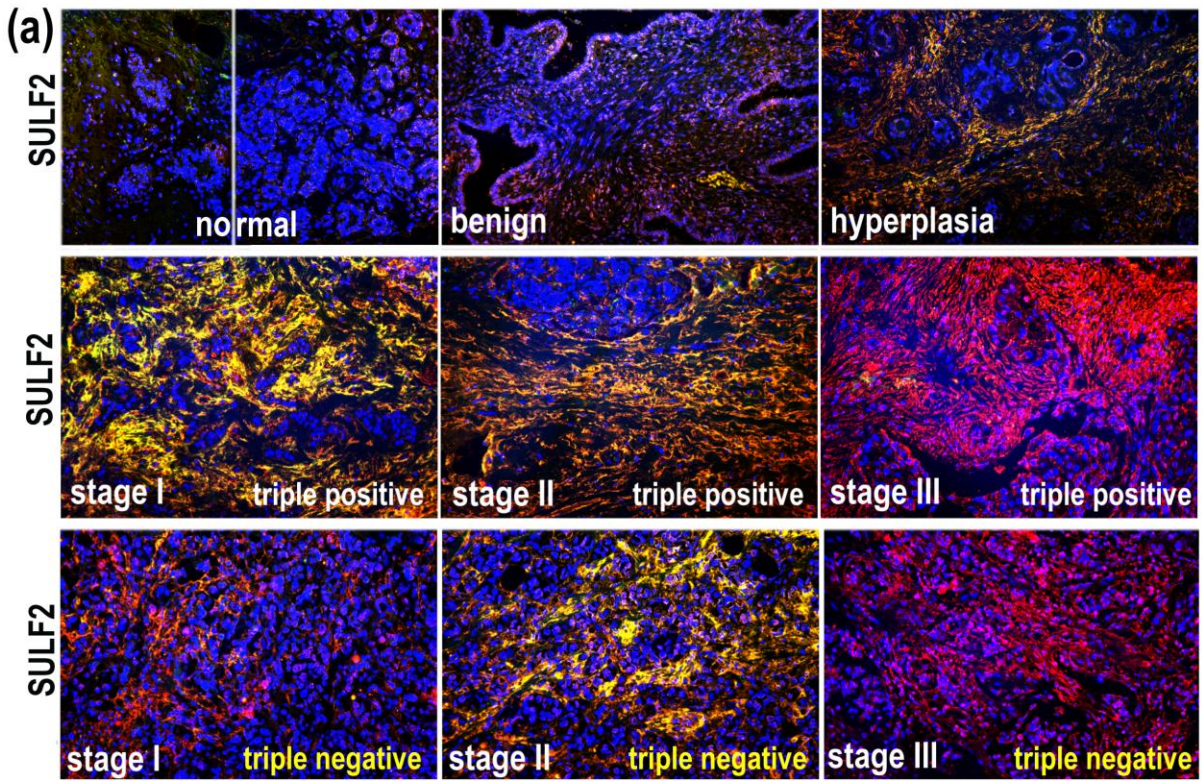

(a)
SULF1
normal
benign
hyperplasia
SULF1
stage I
triple positive
stage II
triple positive
stage III
triple positive
SULF1
stage I
triple negative
stage II
triple negative
stage III
triple negative
(b)
Level of Sulf1 expression
Sulf1-exon 6+
total Sulf1
normal
benign
hyperplasia
stage I
stage II
stage III
stage I
stage II
stage III
triple positive
triple negative
(c)
A antibody
C antibody
Exons 1-5
6
7
8
9-17
18
19-22
Sulf1
Exons 1-5
6
7
8
9-17
18
19-22
Sulf2
N antibody
D antibody

Figure 2:


(a)
SULF2
normal
benign
hyperplasia
SULF2
stage I
triple positive
stage II
triple positive
stage III
triple positive
SULF2
stage I
triple negative
stage II
triple negative
stage III
triple negative

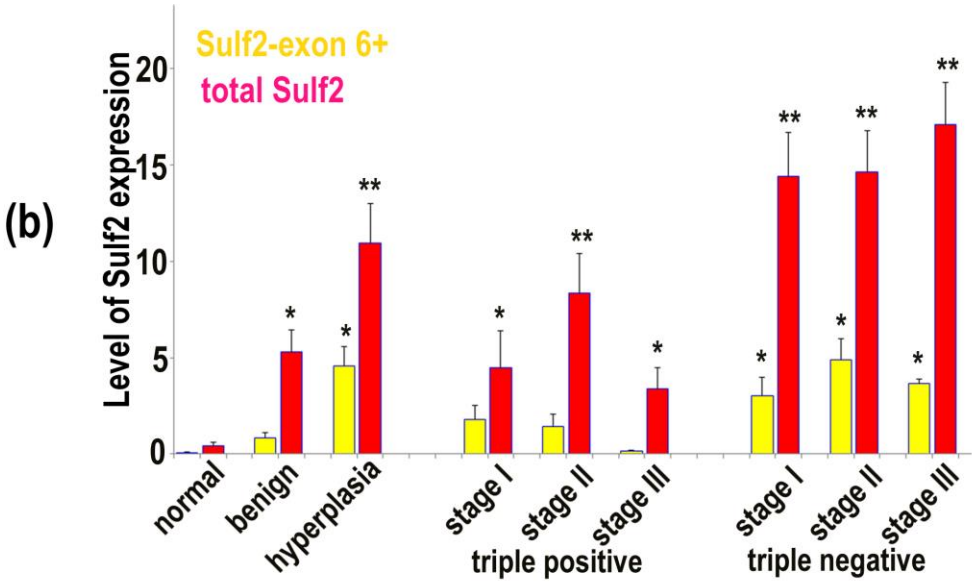

(b)
Sulf2-exon 6+
total Sulf2
Level of Sulf2 expression
0
5
10
15
20
normal
benign
hyperplasia
stage I
stage II
stage III
stage I
stage II
stage III
triple positive
triple negative

Figure 3:

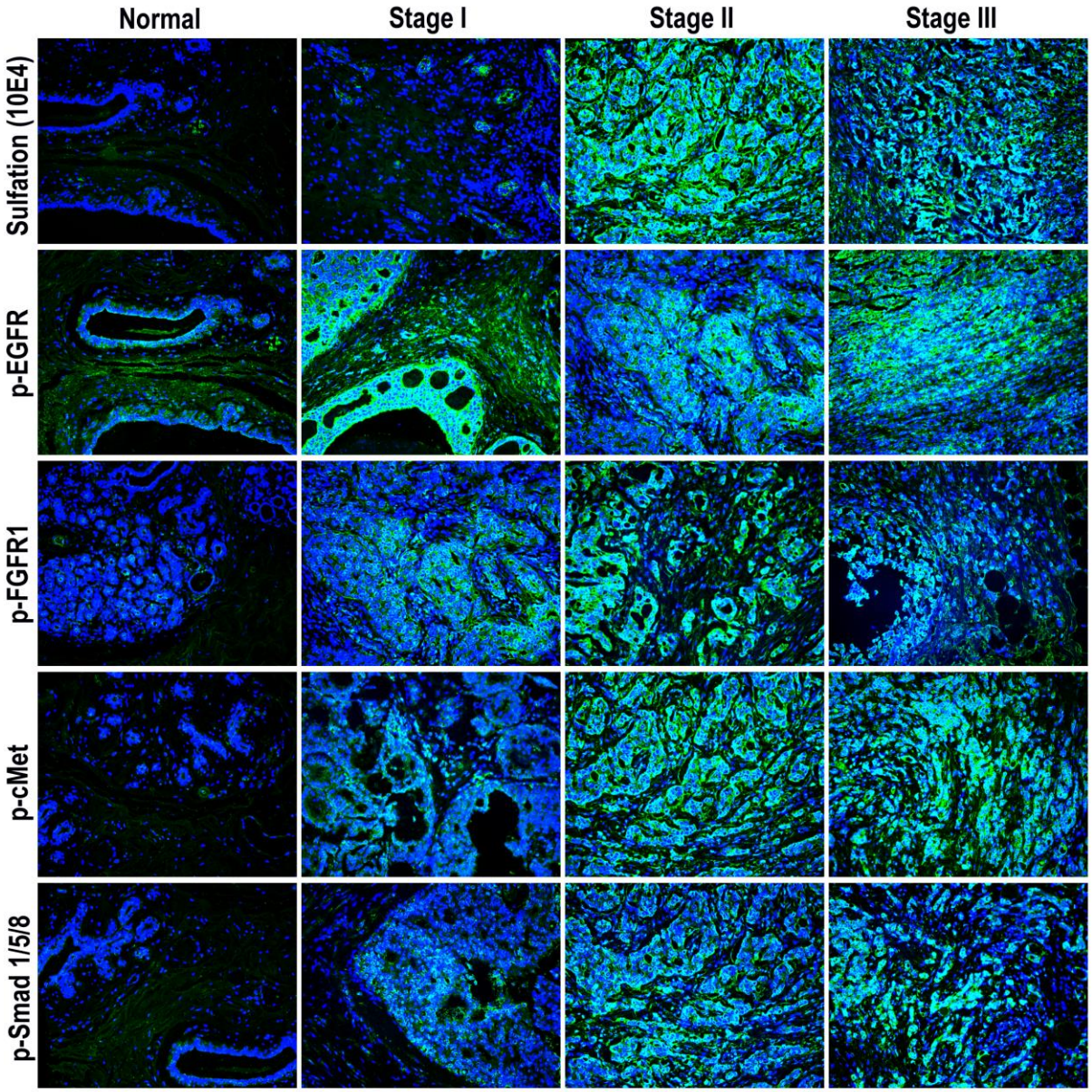

Normal
Stage I
Stage II
Stage III
Sulfation (10E4)
p-EGFR
p-FGFR1
p-cMet
p-Smad 1/5/8

Figure 4:

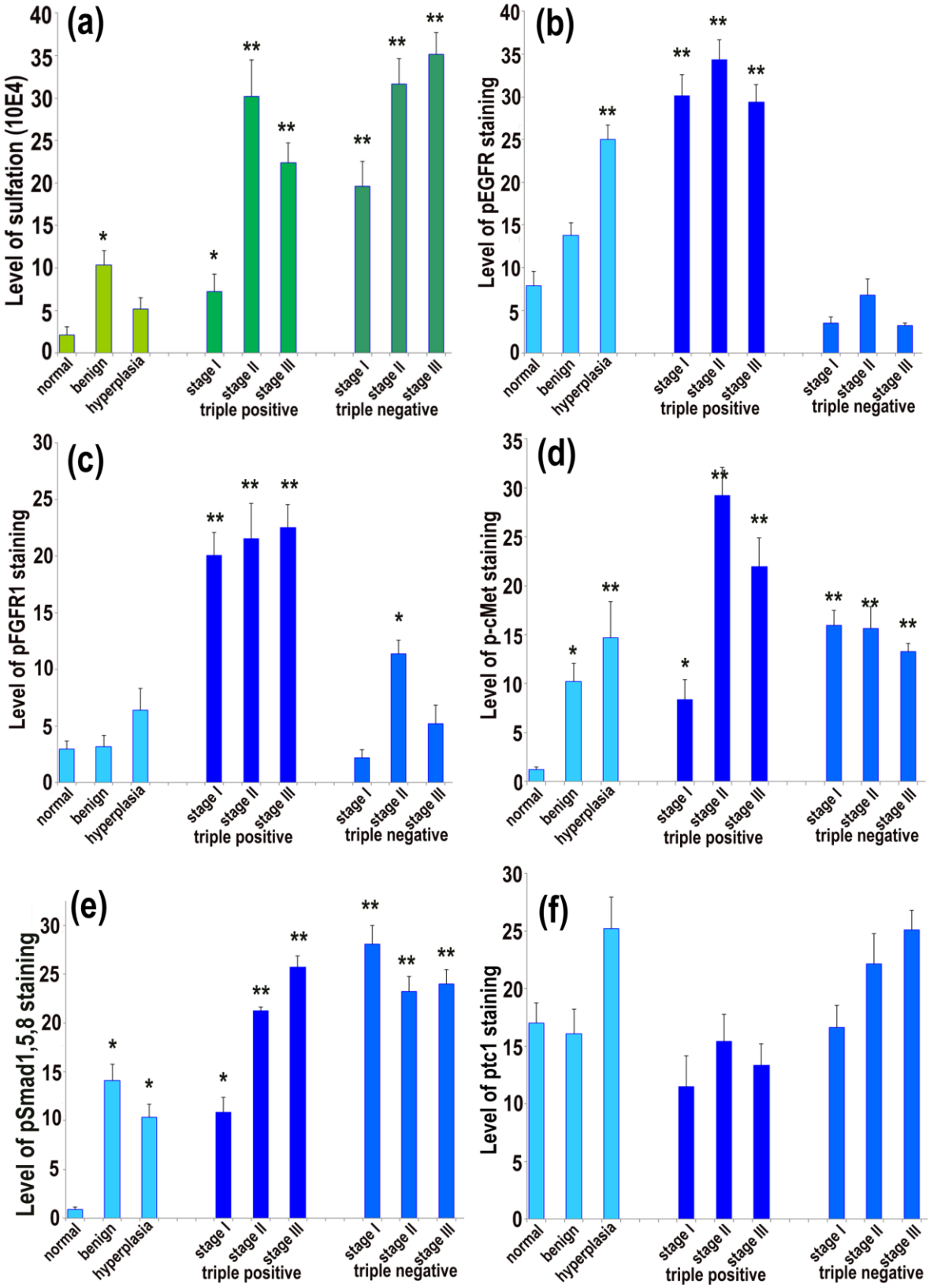

(a)
Level of sulfation (10E4)
(b)
Level of pEGFR staining
(c)
Level of pFGFR1 staining
(d)
Level of p-cMet staining
(e)
Level of pSmad1,5,8 staining
(f)
Level of ptc1 staining
normal
benign
hyperplasia
stage I
stage II
stage III
triple positive
triple negative

Figure 5:

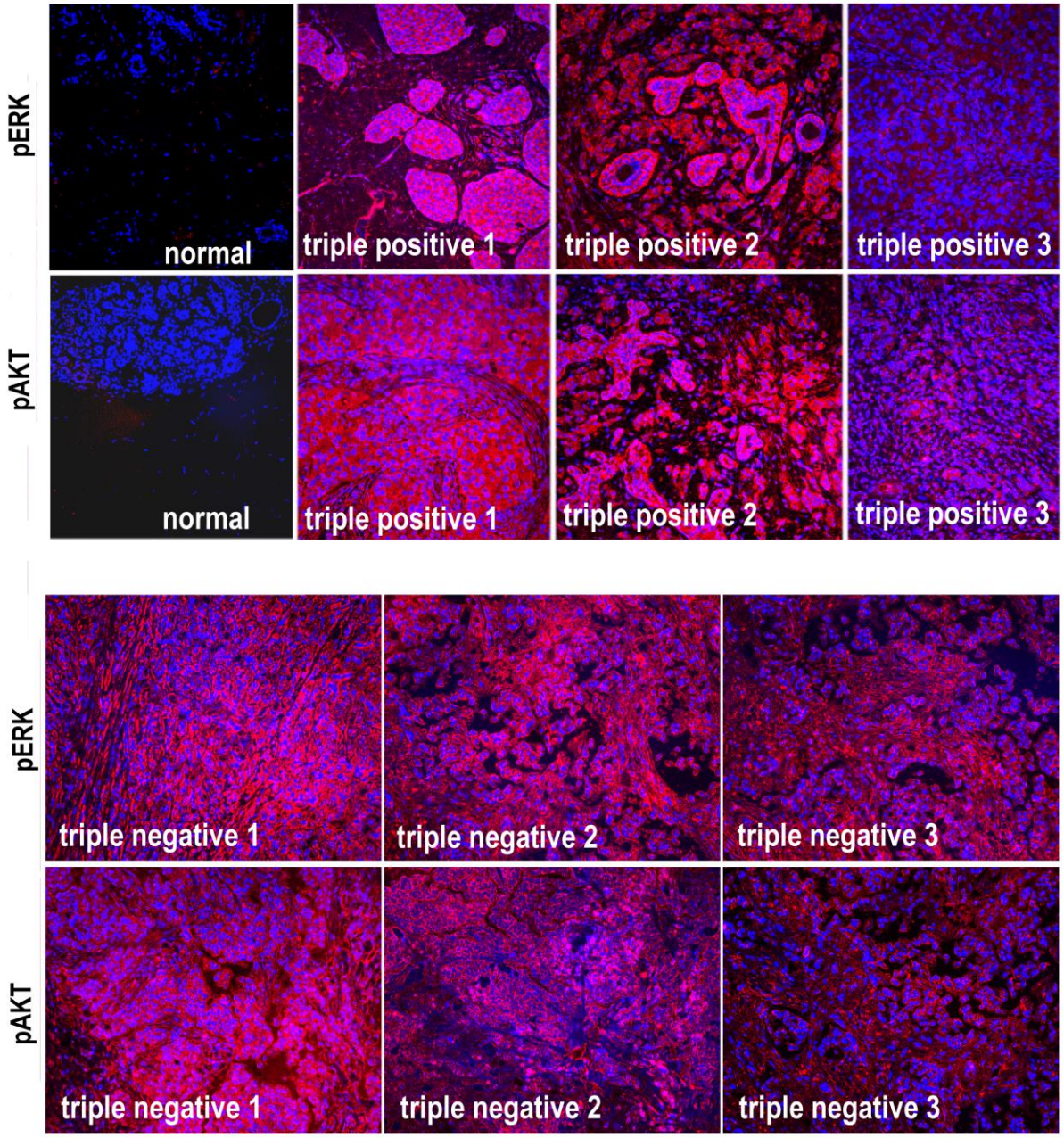

pERK
pAKT
normal
triple positive 1
triple positive 2
triple positive 3
normal
triple positive 1
triple positive 2
triple positive 3
pERK
pAKT
triple negative 1
triple negative 2
triple negative 3
triple negative 1
triple negative 2
triple negative 3

Figure 6:

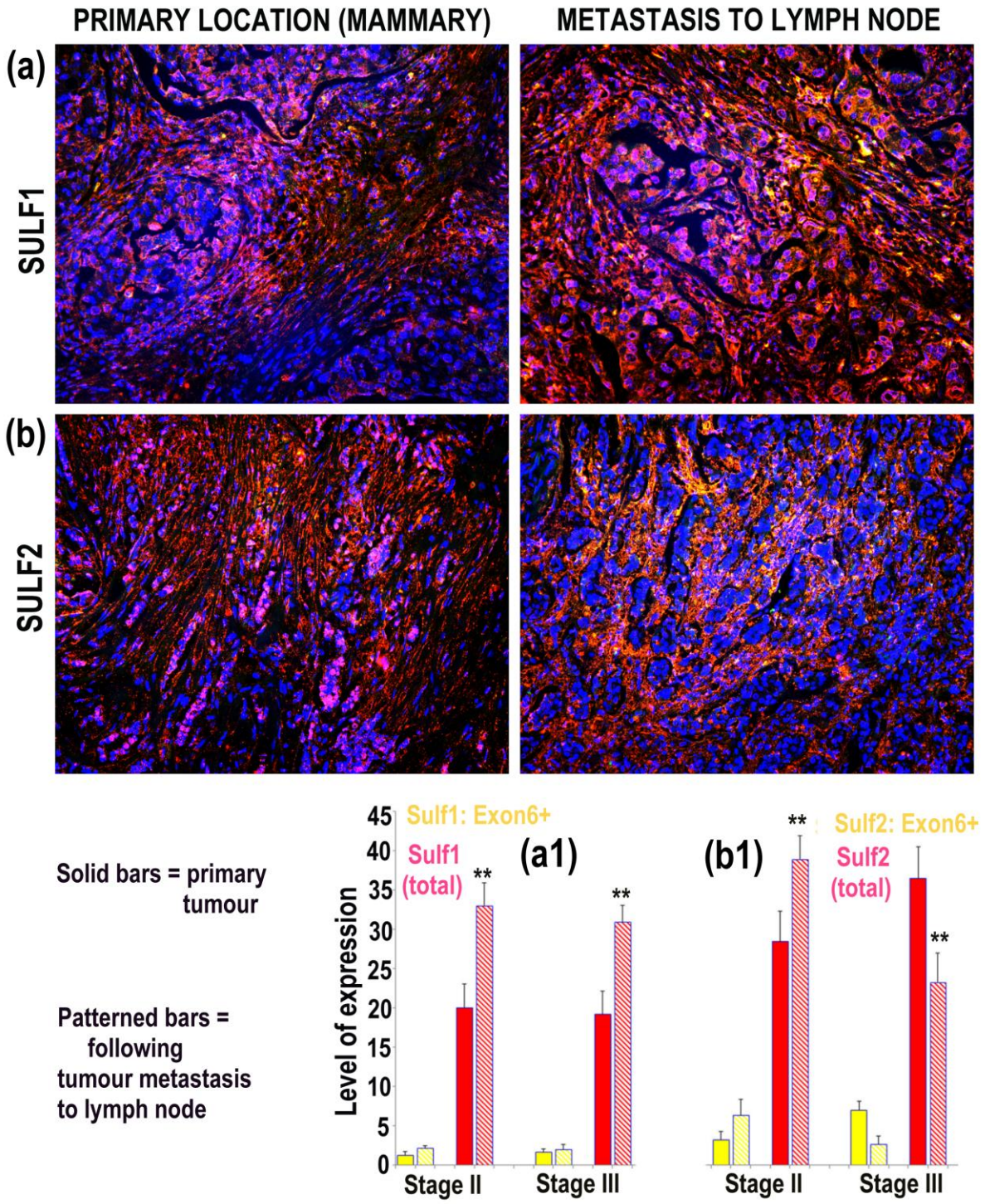

PRIMARY LOCATION (MAMMARY)
METASTASIS TO LYMPH NODE
(a)
SULF1
(b)
SULF2
Solid bars = primary tumour
Patterned bars = following tumour metastasis to lymph node
Level of expression
45
40
35
30
25
20
15
10
5
0
Sulf1: Exon6+
Sulf1 (total)
(a1)
**
**
Stage II
Stage III
Sulf2: Exon6+
Sulf2 (total)
(b1)
**
**
Stage II
Stage III

Figure 7:

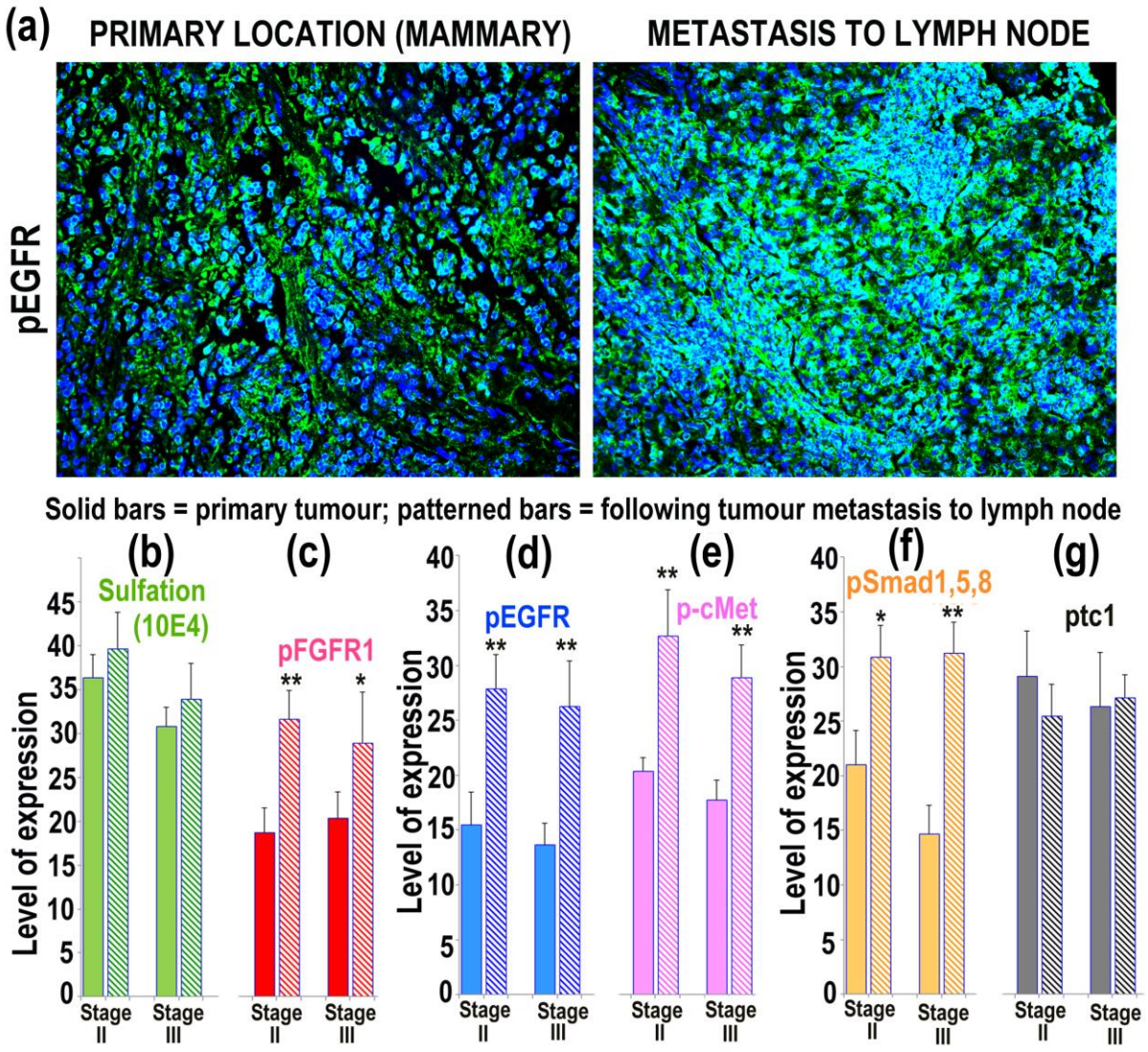
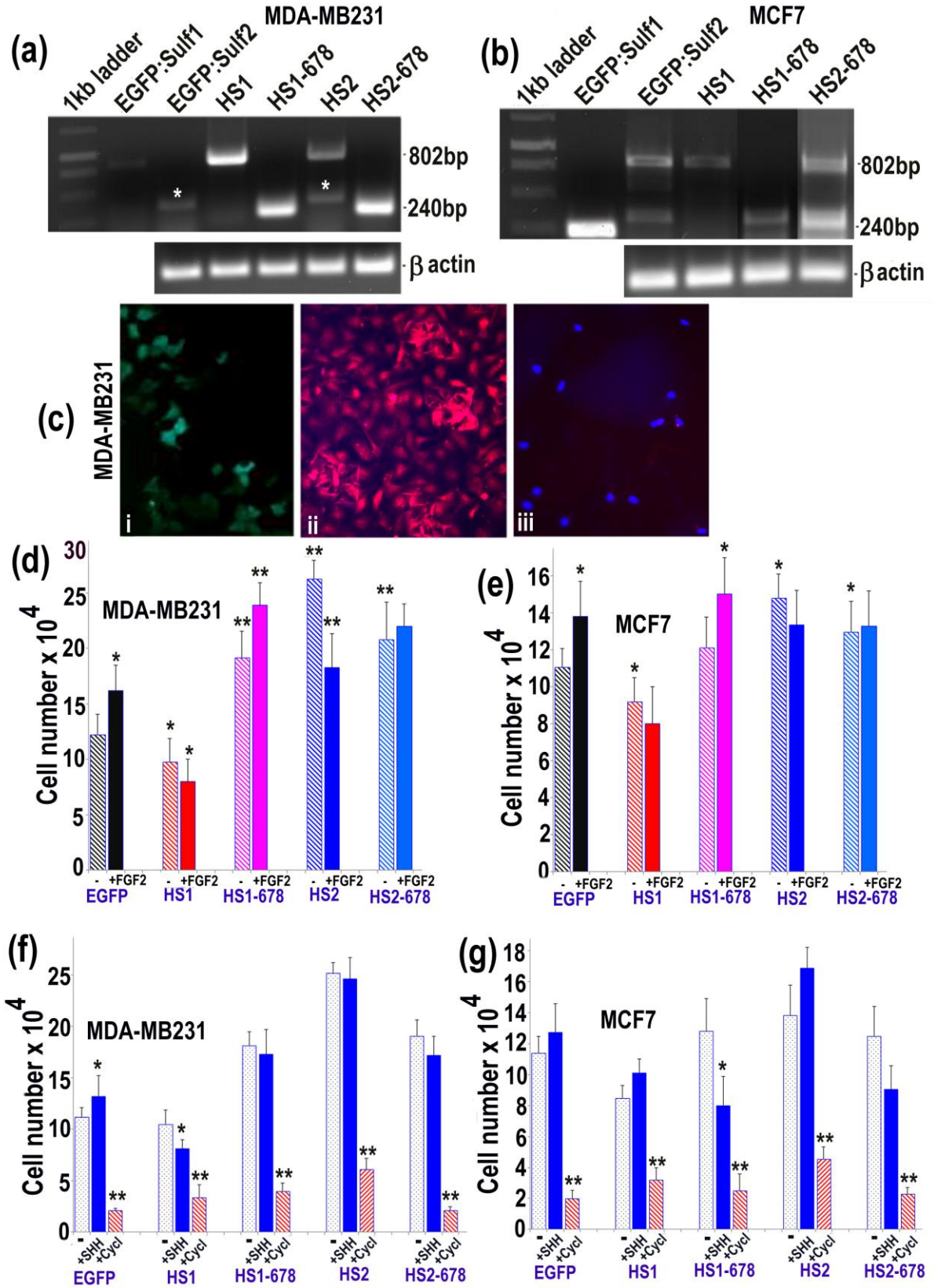

(a)
PRIMARY LOCATION (MAMMARY)
METASTASIS TO LYMPH NODE
pEGFR
Solid bars = primary tumour; patterned bars = following tumour metastasis to lymph node
(b)
Sulfation (10E4)
(c)
pFGFR1
(d)
pEGFR
(e)
p-cMet
(f)
pSmad1,5,8
(g)
ptc1
Level of expression
Stage II
Stage III

Figure 8:


(a)
MDA-MB231
1kb ladder
EGFP:Sulf1
EGFP:Sulf2
HS1
HS1-678
HS2
HS2-678
802bp
240bp
β actin
(b)
MCF7
1kb ladder
EGFP:Sulf1
EGFP:Sulf2
HS1
HS1-678
HS2-678
802bp
240bp
β actin
(c)
MDA-MB231
i
ii
iii
(d)
MDA-MB231
Cell number x 10^4
- +FGF2
EGFP
HS1
HS1-678
HS2
HS2-678
(e)
MCF7
Cell number x 10^4
- +FGF2
EGFP
HS1
HS1-678
HS2
HS2-678
(f)
MDA-MB231
Cell number x 10^4
- +SHH +Cycl
EGFP
HS1
HS1-678
HS2
HS2-678
(g)
MCF7
Cell number x 10^4
- +SHH +Cycl
EGFP
HS1
HS1-678
HS2
HS2-678