Phenotypic Plasticity Determines Cancer Stem Cell Therapeutic Resistance in Oral Squamous Cell Carcinoma

Adrian Biddle a,⁎, Luke Gammon a, Xiao Liang b, Daniela Elena Costea b, Ian C. Mackenzie a

⁎ Corresponding author at: Centre for Cell Biology and Cutaneous Research, Blizard Institute, 4 Newark Street, London E1 2AT, UK. E-mail address: a.biddle@qmul.ac.uk (A. Biddle).

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Abstract
Cancer stem cells (CSCs) drive tumour spread and therapeutic resistance, and can undergo epithelial-to-mesenchymal transition (EMT) and mesenchymal-to-epithelial transition (MET) to switch between epithelial and post-EMT sub-populations. Examining oral squamous cell carcinoma (OSCC), we now show that increased phenotypic plasticity, the ability to undergo EMT/MET, underlies increased CSC therapeutic resistance within both the epithelial and post-EMT sub-populations. The post-EMT CSCs that possess plasticity exhibit particularly enhanced therapeutic resistance and are defined by a CD44highEpCAMlow−/−CD24+ cell surface marker profile. Treatment with TGFβ and retinoic acid (RA) enabled enrichment of this sub-population for therapeutic testing, through which the endoplasmic reticulum (ER) stressor and autophagy inhibitor Thapsigargin was shown to selectively target these cells. Demonstration of the link between phenotypic plasticity and therapeutic resistance, and development of an in vitro method for enrichment of a highly resistant CSC sub-population, provides an opportunity for the development of improved chemotherapeutic agents that can eliminate CSCs.

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1. Introduction
Oral squamous cell carcinoma (OSCC) has an annual worldwide incidence of over 300,000 cases, a mortality rate of 48% (Torre et al., 2015), and commonly develops therapeutic resistance (da Silva et al., 2012). Resistance to therapeutic regimens is a major problem for cancer therapy, as it precludes complete ablation of the tumour and enables local and distant tumour recurrence, the main cause of cancer mortality. Cancer stem cells (CSCs), the sub-population of tumour cells that possess tumour-initiating potential (Clarke et al., 2009; Li et al., 2008) and also drive tumour invasion and metastasis (Charafe-Jauffret et al., 2010; Hermann et al., 2007), have been shown to possess the plasticity required to enable new tumour growth at metastatic sites (Tsai et al., 2012). Therefore, a level of phenotypic plasticity that enables sequential EMT and MET is important to tumour progression (Brabletz, 2012). This phenotypic plasticity is epigenetically regulated (Chaffer et al., 2013; Ke et al., 2010), and not all cells within the epithelial and post-EMT sub-populations possess the plasticity required to enable EMT/MET (Biddle et al., 2011; Chaffer et al., 2013).

We previously identified two distinct CSC sub-populations in OSCC: CD44++EpCAMhigh proliferative epithelial CSCs and CD44highEpCAMlow−/− migratory/metastatic post-EMT CSCs (Biddle et al., 2011). Cells were able to switch between these two sub-populations by undergoing EMT and MET. Equivalent CSC sub-populations have now also been identified in breast cancer (Liu et al., 2014; Sarrio et al., 2012). We also identified a hierarchy of plasticity within the post-EMT CSC sub-population, such that only a subset of post-EMT CSCs could undergo MET to return to an epithelial phenotype (Biddle et al., 2011).

In the present study, we sought to examine the therapeutic resistance of heterogeneous CSC sub-populations in OSCC. We found that cellular
phenotypic plasticity intersects with EMT to determine therapeutic resistance of CSCs. We identified a sub-population of post-EMT CSCs that are highly plastic, resistant to chemotherapeutic drugs including the CSC-targeted therapy (Gupta et al., 2009) salinomycin, and exhibit a CD44highEpCAMlow/CD24+ cell surface marker profile. Their high plasticity and consequent re-establishment of heterogeneity posed a challenge for therapeutic testing, and we therefore developed a co-treatment regime of TGFβ with retinoic acid (RA) for stabilization and enrichment of this sub-population. Gene expression microarray analysis identified up-regulation of processes involved in protein turnover in this sub-population, and this led us to identify Thapsigargin, a sarco/endoplasmic reticulum Ca2+ ATPase (SERCA) inhibitor (Ganey et al., 2011), as a compound that selectively targets these cells. Finally, we demonstrated that fresh OSCC tumour specimens contain CSC sub-populations analogous to those identified in cell lines.

2. Materials and Methods

2.1. Cell Culture

Cell culture, including suspension culture for sphere assays, was performed as previously described (Biddle et al., 2011). The CA1 cell line was previously described (Biddle et al., 2011), and the LM cell line was recently derived in our laboratory from an OSCC of the mandibular region of the mouth. Cell removal from adherent culture was performed using 1 × Trypsin–EDTA (Sigma, T3924) at 37 °C. For TGFβ and RA treatment, cells were plated at a density of 10,000 cells per ml and TGFβ and RA were added to cell culture at the indicated concentrations. Medium and TGFβ and RA were replaced every two days for at least six days and until enough cells were produced for the required assays, whilst ensuring continued sub-confluence. Floating cells in culture are greatly enriched for the post-EMT sub-population; therefore, except for when treated with TGFβ or RA, the floating cells were used at each passage of pEMT-P in order to maintain the post-EMT sub-population.

2.2. Single Cell Cloning

Single cell cloning was performed as previously described (Biddle et al., 2011), using limiting dilution and microscopic examination of wells to identify those that contain a single cell. Clonal sub-lines were maintained under standard tissue culture conditions.

2.3. Flow Cytometry and FACS

Flow cytometry was performed as previously described (Biddle et al., 2011). Antibodies for cell line staining were CD44-PE (clone G44-26, BD Bioscience), CD24-FTTC (clone ML5, BD Bioscience) and EpCAM-APC (clone HEA-125, Miltenyi Biotec). For fresh tumour cells, G44-26, BD Bioscience), CD24-FITC (clone ML5, BD Bioscience) and RA were added to cell culture at the indicated concentrations. Medium and TGFβ and RA were replaced every two days for at least six days and until enough cells were produced for the required assays, whilst ensuring continued sub-confluence. Floating cells in culture are greatly enriched for the post-EMT sub-population; therefore, except for when treated with TGFβ or RA, the floating cells were used at each passage of pEMT-P in order to maintain the post-EMT sub-population.

2.4. Immunofluorescence

FACS sorted tumour cell sub-populations were smeared onto Poly-L-Lysine slides (Thermo Scientific) and incubated at 37 °C for 30 min to promote attachment. Cells were fixed in 4% paraformaldehyde, permeabilised with 0.25% Triton-X (Sigma) in phosphate buffered saline (PBS) (Sigma, D8662), and then blocked overnight in 1% bovine serum albumin (BSA) in PBS. Cells were then incubated overnight with primary antibodies in PBS/1% BSA, washed twice in PBS/1% BSA, and then incubated for 1 h with secondary antibodies in PBS/1% BSA. Cells were washed twice in PBS/1% BSA, incubated for 1 min with DAPI (Sigma) at 1 μg/ml in PBS, washed once in PBS and then mounted with Immu-Mount (Thermo Scientific). Imaging was performed at 200 × magnification, and exposure time was the same for all samples. Images were processed in Adobe Photoshop, with the threshold for high versus low staining the same for all samples. Antibody details can be found in the supplementary information.

2.5. RNA Extraction, cDNA Synthesis and QPCR

RNA extraction, cDNA synthesis and QPCR were performed as previously described (Biddle et al., 2011). Primer sequences are listed in the supplementary information.

2.6. Drug Dose Response Assays

Cells were plated at 1000 cells per well in flat-bottomed 96-well tissue culture plates (Corning). 24 h later, drugs were added at 4 different concentrations in triplicate technical replicates, with triplicate untreated control wells. 72 h after drug addition, cells were fixed in 4% paraformaldehyde and washed in PBS. For automated microscope analysis, cells were permeabilised with 0.1% Triton-X (Sigma) in PBS, then stained with CellMask deep red (Life Technologies H32721, used at 1:30,000 dilution) and 1 μg/ml DAPI (Sigma) for 1 h. Cells were washed twice with PBS. Cell images were acquired using an InCell 1000 automated microscope (GE), and then analysed using InCell Developer Toolbox software (GE) to determine the number of cells. Data was averaged for the triplicate technical replicates and normalized to the untreated wells. Results from at least three independent biological repeat experiments were entered into Graph-Pad Prism software to determine the dose response curve. IC50 and 95% confidence intervals for the IC50, using the nonlinear regression analysis of log(inhibitor) versus response with a variable slope. Drug details can be found in the supplementary information.

2.7. Microarray Analysis

RNA was extracted using the RNeasy microkit (Qiagen) and analysed using an Illumina Human HT-12 v4 gene expression array. The results were analysed using the GenomeStudio software (Illumina), with quantile normalization and a false discovery rate filter of 5% in differential expression analysis. The top 150 differentially expressed genes from each analysis were analysed with the functional annotation clustering tool on the DAVID database (Huang da et al., 2009a, 2009b). Microarray data are deposited in the GEO database under the accession numbers GSE74578 and GSE74580.

2.8. Transplantation Into Immunodeficient Mice

NOD/SCID mice were obtained from Jackson Laboratories. Mice used in this study were of mixed gender and older than 6 weeks of age. The mice were maintained in a certified isolation facility under a pathogen free environment with standard 12/12 h day and night cycle, in accordance with European guidelines. All animal procedures were approved by the Norwegian Animal Research Authority. Cells were harvested from adherent culture and resuspended in 50 μl of Matrigel (BD Biosciences) on ice. The suspension was injected orthotopically into the tongues of NOD/SCID mice. Tumours were detected by palpation and the tumour volume was manually assessed with a digital calliper.

2.9. Isolation of Cells From Human Tumours

Tumour specimens were obtained from the pathology department at Barts Health NHS Trust, with full local ethical approval and patients’ informed consent. Specimen site was selected to avoid both the tumour margin and necrotic core, and specimens were kept overnight at 4 °C in epithelial growth medium (termed FAD) with 10% FBS (Locke et al., 2005). Specimens were washed in PBS to remove blood, minced into approximately 1 mm3 pieces using scalpels, and then incubated with...
We examined survival of CD44highEpCAMlow/3.1. CSC Plasticity Intersects With EMT to Determine Therapeutic Resistance

3. Results

3.1. CSC Plasticity Intersects With EMT to Determine Therapeutic Resistance

We initially examined the therapeutic resistance of post-EMT CSCs. We examined survival of CD44highEpCAMlow/ post-EMT CSCs in the CA1 and LM OSCC cell lines using two chemotherapeutic agents, paclitaxel and cisplatin (Fig. 1A). The post-EMT CSCs preferentially survived paclitaxel treatment, but showed no preferential survival of cisplatin treatment. This indicates that cisplatin resistance is not driven by EMT, and raises the question of whether resistance to cisplatin depends on alternative CSC sub-populations.

To investigate whether CSC plasticity could influence therapeutic response, we generated four clonal CA1 sub-lines which differ in their possession of phenotypic plasticity and was lost as the clonal sub-lines passed through 10 passages. To determine whether differences in plasticity could influence therapeutic response, we generated four clonal CA1 sub-lines which differ in their possession of phenotypic plasticity and was lost as the clonal sub-lines passed through 10 passages.

We next sought to block the loss of plasticity that occurs over extended passage (Fig. S1F and S1G) in concert with loss of plasticity and drug resistance. The CD24− fraction from pEMT-P was enriched in the ability to undergo MET, relative to the CD24+ pEMT-P fraction (Fig. 1D), confirming CD24 as a marker of plasticity. To determine the contribution of the CD44highEpCAMlow/CD24+ plastic post-EMT CSCs to therapeutic resistance, we examined their survival within the post-EMT sub-population in the CA1 and LM OSCC cell lines upon treatment with paclitaxel or cisplatin (Fig. 1E). The CD24+ plastic post-EMT CSCs preferentially survived cisplatin treatment, but showed no preferential survival of paclitaxel treatment compared to the rest of the post-EMT sub-population. This was in agreement with the findings above showing that post-EMT CSC sub-populations are resistant to paclitaxel irrespective of plasticity, whereas plasticity is important for resistance to cisplatin (Fig. 1F).

To determine if the four clonal sub-lines had different in vivo tumour initiating properties, we performed orthotopic xenograft studies in NOD/SCID mice. Injecting 5000 cells from Epi-S, Epi-P or pEMT-P produced tumours in 6/6, 5/6 and 5/6 mice respectively, whereas only 1 mouse out of 6 formed a tumour when injected with pEMT-S (Fig. S1H). Epi-S and the parental CA1 line both produced fast-growing tumours, Epi-P and pEMT-P produced slower-growing tumours, and the single tumour produced by Epi-S only grew to a very small size (Fig. S1I). Importantly, pEMT-S produced the greatest number of tumourspheres in suspension culture (Fig. S1J), an assay of self-renewal that biases towards self-renewing post-EMT cells (Biddle et al., 2011; Chaffer et al., 2006). This supports the notion that the pEMT-S sub-population is not deficient in the ability to self-renew.

3.2. Co-treatment With TGFβ and RA Maintains Plasticity and Therapeutic Resistance

We next sought to block the loss of plasticity that occurs over extended passage in pEMT-P, in order to determine whether this would also block the loss of cisplatin resistance. A method for experimental maintenance of plastic post-EMT CSCs underlies its inability to initiate tumour formation.

We investigated whether differences in plasticity could influence therapeutic response by assessing the responses of the four clonal sub-lines to the chemotherapeutic drugs paclitaxel and cisplatin (Figs. 1B and C and S1E). Salinomycin, an antibiotic previously reported to target the EMT markers Vimentin and Zeb1 and downregulated expression of the epithelial markers E-cadherin and Keratin 15. The transcription factor Prrx1, which prevents MET (Ocana et al., 2012), was highly expressed in pEMT-S. Expression of Calgranulin B, a marker of epithelial terminal differentiation (Martinsson et al., 2005), was lost in all four clonal sub-lines. These data demonstrate that both drug sensitive and drug resistant sub-populations contain self-renewing CSCs. There is evidence that post-EMT CSCs must undergo MET in order to drive new tumour formation (Ocana et al., 2012; Tsai et al., 2012), and our data suggest that the lack of plasticity of the pEMT-S sub-line undermines its inability to initiate tumour formation.

We next sought to identify a cell surface marker that could be used to detect plastic CSCs in flow cytometry. CD24 was identified as a potential marker of plasticity from gene expression microarray analysis comparing Epi-P to Epi-S and pEMT-P to pEMT-S. The percentage of CD24+ cells was higher in flow cytometric analysis of Epi-P and pEMT-P compared to Epi-S and pEMT-S, respectively, and was reduced over extended passage (Fig. S1F and S1G) in concert with loss of plasticity and drug resistance. The CD24− fraction from pEMT-P was enriched in the ability to undergo MET, relative to the CD24− pEMT-P fraction (Fig. 1D), confirming CD24 as a marker of plasticity. To determine the contribution of the CD44highEpCAMlow/CD24− plastic post-EMT CSCs to therapeutic resistance, we examined their survival within the post-EMT sub-population in the CA1 and LM OSCC cell lines upon treatment with paclitaxel or cisplatin (Fig. 1E). The CD24− plastic post-EMT CSCs preferentially survived cisplatin treatment, but showed no preferential survival of paclitaxel treatment compared to the rest of the post-EMT sub-population. This was in agreement with the findings above showing that post-EMT CSC sub-populations are resistant to paclitaxel irrespective of plasticity, whereas plasticity is important for resistance to cisplatin (Fig. 1F).

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3.2. Co-treatment With TGFβ and RA Maintains Plasticity and Therapeutic Resistance

We next sought to block the loss of plasticity that occurs over extended passage in pEMT-P, in order to determine whether this would also block the loss of cisplatin resistance. A method for experimental maintenance of cells in the plastic post-EMT sub-population would also be advantageous for therapeutic testing, as this sub-population exhibits resistance to all drugs tested so far. TGFβ induces EMT (Biddle et al., 2011), but extended TGFβ treatment drove the CA1 post-EMT sub-population into a stable post-EMT state (Fig. S2A). Contrastingly, RA induces epithelial differentiation (Metallo et al., 2008) and opposes TGFβ activity during development (Chen et al., 2007). We therefore tested whether RA could block the transition of pEMT-P into a pEMT-S-like state and act in concert with TGFβ to restrain cells in the plastic post-EMT sub-population. 5 μM RA caused increased MET of pEMT-P, creating a mixed population of epithelial and post-EMT cells, and had no effect on pEMT-S. The effect of RA was abrogated by 5 ng/ml TGFβ and did not prevent 5 ng/ml TGFβ from driving cells into a stable post-EMT state (Fig. S2B and S2C). However, by titrating the TGFβ dose we were able to establish a co-treatment regime that enabled maintenance of the plastic post-EMT sub-population (0.5 ng/ml TGFβ in combination with 5 μM RA) (Fig. S2D and S2E). Importantly, treatment of pEMT-P with 0.5 ng/ml TGFβ and
5 μM RA maintained this sub-line in the CD44<sup>high</sup>EpCAM<sup>low−</sup>CD24<sup>+</sup> state (Figs. 2A and B and S2F and S2H). pEMT-P retained the ability to undergo MET upon treatment withdrawal (Fig. 2C), indicating maintenance of plasticity. Resistance to cisplatin and paclitaxel was maintained in pEMT-P treated with TGFβ and RA (Figs. 2D and S2I), demonstrating that maintained CSC plasticity results in maintenance of drug resistance.

To validate this protocol, we tested it using a second cell line. We FACS sorted the CD44<sup>high</sup>EpCAM<sup>low−</sup>CD24<sup>+</sup> fraction from the LM OSCC cell line and treated the cells with TGFβ and RA during population expansion in culture. Treatment with TGFβ and RA maintained the cells in a CD44<sup>high</sup>EpCAM<sup>low−</sup>CD24<sup>+</sup> state (Figs. 2E and F and S2G and S2H). The ability to undergo MET upon treatment withdrawal was also maintained (Fig. 2G), as was resistance to cisplatin and paclitaxel (Figs. 2H and S2J). Therefore, plasticity can be maintained in FACS sorted CD44<sup>high</sup>EpCAM<sup>low−</sup>CD24<sup>+</sup> cells and this results in maintenance of drug resistance. In summary, we have developed a TGFβ + RA protocol for the enrichment of the plastic post-EMT CSC sub-population which dispenses with the need for single cell cloning experiments.

3.3. Thapsigargin Specifically Targets the Drug Resistant Plastic Post-EMT CSCs

To identify targetable cellular processes for therapeutic testing, we performed functional annotation clustering analysis of gene expression...
microarray data for genes upregulated in plastic sub-lines (Epi-P and pEMT-P compared to Epi-S and pEMT-S) and for genes upregulated in the TGFβ+ RA protocol for retention of plasticity (TGFβ+R At r e a t e d pEMT-P and CD44highEpCAMlow/−CD24+ LM fraction compared to control and TGFβ treatments alone) (Tables S1–S3). Multiple cellular processes were upregulated in all three analyses, predominantly
biosynthetic processes such as intracellular organelle production (including lysosomes and other membrane-bound organelles) and protein complex biogenesis, indicating high protein turnover. These processes may be required for the cellular remodelling intrinsic to phenotypic plasticity, and we attempted to sensitize the plastic post-EMT sub-population to cell death by targeting processes involved in protein turnover in TGF\(\beta\)+ RA treated pEMT-P and CD44\textsuperscript{high}EpCAM\textsuperscript{low}/CD24\textsuperscript{+} LM fraction. CX-5461, a selective inhibitor of RNApol1 (Drygin et al., 2011), did not cause increased death of the plastic post-EMT sub-population either alone or in combination with cisplatin (Fig. S3A–S3C). Bafilomycin A1, an inhibitor of lysosomal fusion (Yamamoto et al., 1998), and Tunicamycin, an inhibitor of protein folding in the endoplasmic reticulum (ER) that induces the ER unfolded protein response (UPR) (Xu et al., 2005), both induced increased death of post-EMT cells compared to the CA1 and LM parental lines (Figs. 3A and B and S3D and S3E). However, they did not specifically target the plastic post-EMT subpopulation and targeting of post-EMT cells was weak. Thapsigargin, a SERCA inhibitor that both induces the ER UPR and inhibits lysosomal fusion (Ganley et al., 2011), specifically targeted the plastic post-EMT subpopulation in both the CA1 and LM lines (Figs. 3A and B and S3D and S3E). Therefore, the TGF\(\beta\)+ RA protocol can be used to identify compounds that target the plastic post-EMT CSC sub-population, and we have identified Thapsigargin as one such compound.

3.4. OSCC Tumour Specimens Contain CSC Sub-populations With Corresponding Properties To Those Identified in Cell Lines

We next investigated whether CSC sub-populations analogous to those identified in cell lines exist in fresh clinical specimens of OSCC. The distribution of CD44, EpCAM and CD24 staining on the specimen FACS plots corresponded with that seen in cell lines (Figs. 4Aa and Bb and S4A). Cells freshly isolated from OSCC tumours survive poorly ex vivo, so we assessed their attributes by direct antibody staining of FACS sorted post-EMT CD44\textsuperscript{high}EpCAM\textsuperscript{low}/CD24\textsuperscript{+} and CD44\textsuperscript{high}EpCAM\textsuperscript{low}/CD24\textsuperscript{−} fractions and epithelial CD44\textsuperscript{+}EpCAM\textsuperscript{high}CD24\textsuperscript{−} and CD44\textsuperscript{+}EpCAM\textsuperscript{high}CD24\textsuperscript{−} fractions from 7 specimens (Figs. 4Ca and S4B–S4H). Both the post-EMT CD44\textsuperscript{high}EpCAM\textsuperscript{low}/CD24\textsuperscript{+} and CD44\textsuperscript{high}EpCAM\textsuperscript{low}/CD24\textsuperscript{−} fractions stained highly for vimentin and low for pan-keratin, whereas both the epithelial CD44\textsuperscript{+}EpCAM\textsuperscript{high}CD24\textsuperscript{−} fractions stained highly for vimentin and low for pan-keratin, whereas both the epithelial CD44\textsuperscript{+}EpCAM\textsuperscript{high}CD24\textsuperscript{−} fractions stained highly for vimentin and low for pan-keratin, whereas both the epithelial CD44\textsuperscript{+}EpCAM\textsuperscript{high}CD24\textsuperscript{−} fractions stained highly for vimentin and low for pan-keratin. This confirmed their post-EMT and epithelial identities, respectively. Activated caspase-3, a marker of cells entering apoptosis, was used to assess sensitivity to cell death. It was most highly expressed in the epithelial CD24\textsuperscript{−} fraction, had lowest expression in the post-EMT CD24\textsuperscript{+} fraction, and was expressed at an intermediate level in the epithelial CD24\textsuperscript{−} and post-EMT CD24\textsuperscript{−} fractions. This was in agreement with the therapeutic sensitivities of the corresponding cell line sub-populations. Lysosomal
processes were important to plastic sub-populations in cell lines, so we stained tumour fractions for the lysosome marker LAMP-2. LAMP-2 was more highly expressed in the post-EMT CD24+ and epithelial CD24+ fractions than in either CD24− fraction, again in agreement with the cell line data. These data indicate that CSC sub-populations exist in fresh tumour specimens with corresponding properties to those identified in cell lines.

4. Discussion

There has recently been considerable focus on the role of CSCs in tumour therapeutic resistance (Gupta et al., 2009; Li et al., 2008) but, despite the existence of heterogeneous CSC phenotypes (Biddle et al., 2011; Hermann et al., 2007; Liu et al., 2014), differences in therapeutic resistance between CSC sub-populations have not previously been investigated. We find that differing phenotypic plasticity is a key determinant of differences in therapeutic resistance between CSC sub-populations. Given the importance of phenotypic plasticity in tumour invasion and metastasis (Brabletz, 2012; Tsai et al., 2012), a sub-population of CSCs that exhibit both phenotypic plasticity and therapeutic resistance presents a potent threat and is likely to be an important target for therapeutic intervention. Our development of a method for enrichment of this most resistant CSC sub-population through co-treatment with TGFβ3 and RA provides a means for producing large numbers of these cells for drug development studies. This sub-population possesses a CD44+ EpCAMlow CD24+ cell surface marker profile, and can therefore be monitored by flow cytometric analysis. As a proof of principle, we performed a targeted study and identified the SERCA inhibitor Thapsigargin as a compound to which this sub-population is selectively sensitive, demonstrating the potential of this method for therapeutic development. Identification of corresponding CSC sub-populations with corresponding resistance to cell death in fresh clinical specimens of OSCC indicates that this is an important new aspect of cancer biology with relevance to human tumours.

The precise mechanism linking plasticity with drug resistance remains to be determined. In this study, we identified the importance to plastic CSCs of processes involved in protein turnover. This suggests that rapid turnover of cellular contents might be required for cellular remodelling during transitions between phenotypic states, for which plastic CSCs are prepared. This rapid turnover is likely to exert considerable cellular stress, and the observed therapeutic resistance might be a by-product of mechanisms intended to protect the cell from stress during phenotypic transitions (Buchberger et al., 2010; Kroemer et al., 2010). We observed that plastic CSCs are extremely sensitive to Thapsigargin, which targets both lysosomal degradation and ER protein processing (Ganley et al., 2011). Interfering with ER protein processing would be expected to cause accumulation of misfolded proteins, and simultaneous inhibition of lysosomal degradation might prevent degradation of misfolded proteins so that they accumulate and overwhelm the cell. Plastic CSCs, undergoing rapid protein turnover, might be particularly sensitive to these perturbations.

In this study, CD24 was identified as a marker of plastic CSC sub-populations. CD24 is a mucin-like adhesion molecule expressed on the cell surface of multiple different cell types through a glycosylphosphatidylinositol membrane anchor, and high CD24 expression has been associated with more aggressive disease in ovarian, breast, lung and prostate cancer (Kristiansen et al., 2004). CD24 is a ligand for endothelial cell P-selectin, suggesting that it may act in metastatic dissemination of tumour cells (Aigner et al., 1997), and it has been shown to promote both tumour cell invasion (Bretz et al., 2012) and metastasis (Lau et al., 2014). CD24 was initially identified as a negative marker for CSCs in breast cancer (Al-Hajj et al., 2003), but has more recently been described as a positive marker in lung cancer (Lau et al., 2014), colorectal cancer (Yeung et al., 2010), and triple-negative breast cancer (Azzam et al., 2013). It has been shown to mark a transient chemoresistant cell state in lung cancer (Sharma et al., 2010) and breast cancer (Goldman et al., 2015). Furthermore, CD24 has been implicated in regulation of EMT (Lim et al., 2014). Further studies into a potential role for CD24 in the control of CSC therapeutic resistance and plasticity may elucidate downstream regulatory mechanisms and suggest new therapeutic targets.

The analysis of fresh clinical specimens in this study provides a first indication that our in vitro model accurately models sub-populations existing in human tumours. However, to fully realise the potential of our in vitro model system for therapeutic development, future studies should further investigate its relevance to the in vivo disease state. Other tumour models, utilising fresh human tumour specimens, will need to be utilised to confirm the therapeutic responses of CSC sub-populations. It will also be important to investigate both a) the power of markers of plastic CSC sub-populations for predicting patient therapeutic response and b) the effect of clinical interventions in human subjects on markers of plastic CSC sub-populations.

This study has demonstrated that phenotypic plasticity underlies CSC therapeutic resistance. The demonstration that a highly plastic and drug resistant CSC sub-population can be stabilized by balancing opposing signalling pathways, and thus enriched in vitro, provides an opportunity for the development of novel therapeutic strategies targeting drug resistant CSCs that may otherwise survive therapeutic intervention and drive tumour recurrence. Given the similar underlying biology of epithelial tumours, these findings are likely to also be relevant to other cancers.
Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ebiom.2016.01.007.

Conflict of Interest

The authors declare no conflicts of interest.

Author Contributions

A.B. and I.C.M. designed the study and wrote the manuscript; A.B. performed the experiments; L.G. provided technical support and performed the automated microscope analysis; X.L. and D.E.C. performed the mouse transplantation experiments.

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References

Aigner, S., Stoeber, Z.M., Fogel, M., Weber, E., Zarn, J., Ruppert, M., Zeller, Y., Vestweber, D., Stahl, R., Sammar, M., Alttevogt, P., 1997. CD24, a mucin-type glycoprotein, is a ligand for P-selectin on human tumor cells. Blood 89, 3385–3395.

Al-Hajj, M., Wicha, M.S., Benito-Hernandez, A., Morrison, S.J., Clarke, M.F., 2003. Prospective identification of tumorigenic breast cancer cells. Proc. Natl. Acad. Sci. U. S. A. 100, 3983–3988.

Azzam, D.J., Zhao, D., Sun, J., Minn, A.J., Ranganathan, P., Drews-Eiger, K., Han, X., Picon-Ruiz, M., Gilbert, C.A., Wunder, S.A., et al., 2013. Triple-negative breast cancer initiating cell subsets differ in functional and molecular characteristics and in gamma-secretase inhibitor drug responses. EMBO Mol. Med. 5, 1502–1522.

Biddle, A., Liang, X., Gammon, L., Faciz, B., Harper, L.J., Emrich, H., Costea, D.E., Mackenzie, L.C., 2011. Cancer stem cells in squamous cell carcinoma switch between two distinct phenotypes that are preferentially migratory or proliferative. Cancer Res. 71, 5317–5326.

Brabletz, T., 2012. EMT and MET in metastasis: where are the cancer stem cells? Cancer Cell 22, 699–701.

Bretz, N., Noske, A., Keller, S., Erbe-Hofmann, N., Schlange, T., Salnikov, A.V., Moldenhauer, G., Kristiansen, G., Alttevogt, P., 2012. CD24 promotes tumour cell invasion by suppressing tissue factor pathway inhibitor-2 (TFPI-2) in a c-Src-dependent fashion. Clin. Exp. Metastasis 29, 27–38.

Buenger, A., Bukau, B., Sommer, T., 2010. Protein quality control in the cytosol and the endoplasmic reticulum: brothers in arms. Mol. Cell 40, 238–252.

Chaffer, C.L., Brennan, J.P., Slavin, J.L., Blick, T., Thompson, E.W., Williams, E.D., 2006. Mesenchymal-to-epithelial transition facilitates bladder cancer metastasis: role of fibroblast growth factor receptor-2. Cancer Res. 66, 11271–11278.

Chaffer, C.L., Marjanovic, N.D., Lee, T., Bell, G., Kleer, C.G., Reinhardt, F., D’Alessio, A.C., Young, R.A., Weinberg, R.A., 2013. Poised chromatin at the ZEB1 promoter enables survival. Proc. Natl. Acad. Sci. U. S. A. 107, 11243–11248.

Cho, M.H., Choe, J., Lee, D.H., Kim, D.Y., Yoon, S.H., Oh, J.H., Lee, Y., Shin, Y., et al., 2008. Intrinsic resistance of tumorigenic breast cancer cells to chemotherapy. J. Natl. Cancer Inst. 100, 672–679.

Chim, Y., Sato, J., Isono, M., Watanabe, S., Takeda, K., Koyama, K., Iizuka, N., et al., 2007. Global screening of selective inhibitors of cancer stem cells by high-throughput anticancer drug discovery. EMBO J. 26, 488–491.

Chen, F., Desai, T.J., Qian, J., Niederreither, K., Lu, J., Cardoso, W.V., 2007. Inhibition of TGF-beta-mediated epithelial-mesenchymal transition as a therapeutic target in oral squamous cell carcinoma. J. Pathol. 236, 505–516.

Ke, X.S., Qu, Y., Cheng, Y., Li W.C., Rotter, V., Oyan, A.M., Kalland, K.H., 2010. Global profiling of histone and DNA methylation reveals epigenetic-based regulation of gene expression during epithelial to mesenchymal transition in prostate cells. BMC Genomics 11, 6139.

Kreso, A., O’Brien, C.A., van Galen, P., Gao, G.L., Notta, F., Brown, A.M., Ng, K., Ma, J., Willoughby, E., Dunant, C., et al., 2013. Variable clonal repopulation dynamics influences chemotherapeutic response in colorectal cancer. Science 339, 543–548.

Kristiansen, G., Sammar, M., Alttevogt, P., 2004. Tumour biological aspects of CD24, a mucin-like adhesion molecule. J. Mol. Histol. 35, 255–262.

Kroener, A., Marino, G., Levine, B., 2010. Autophagy and the integrated stress response. Mol. Cell 40, 280–293.

Lau, A.N., Curtis, S.J., Fillmore, C.M., Rowthorn, S.P., Mosheni, M., Wagner, D.E., Beere, A.M., Montoro, D.T., Sinkevics, K.W., Walton, Z.L., et al., 2014. Tumor-propagating cells and Yap-Taz activity contribute to lung tumor progression and metastasis. EMBO J. 33, 468–481.

Li, X., Lewis, M.T., Huang, J., Gutierrez, C., Osborne, C.K., Wu, M.F., Hilsenbeck, S.G., Pavlick, A., Zhang, Xe, Charness, G.C., et al., 2008. Intrinsic resistance of tumorigenic breast cancer cells to chemotherapy. J. Natl. Cancer Inst. 100, 672–679.

Lim, J., Lee, K.M., Shim, Jin, Sh., 2014. CD24 regulates stemness and the epithelial to mesenchymal transition through modulation of Notch1 mRNA stability by p38MAPK. Arch. Biochem. Biophys. 558, 120–126.

Liu, S., Cong, Y., Wang, D., Sun, Y., Deng, L., Liu, Y., Martin-Trevino, R., Shang, L., McDermott, S.P., Landis, M.D., et al., 2014. Breast cancer stem cell transition between epithelial and mesenchymal states reflective of their normal counterparts. Stem Cell Rep. 2, 78–91.

Locke, M., Heywood, M., Fawell, S., Mackenzie, I.C., 2005. Retention of intrinsic stem cell hierarchies in carcinoma-derived cell lines. Cancer Res. 65, 8944–8950.

Martinsson, H., Yhr, M., Enerback, C., 2005. Expression patterns of S100A7 (pontin) and S100A5 (calgranulin B) in keratinocyte differentiation. Exp. Dermatol. 14, 161–168.

Metallo, C.M., J., De Pablo, J.J., Palecek, S.P., 2008. Retinoic acid and bone morphogenetic protein signaling synergize to efficiently direct epithelial differentiation of human embryonic stem cells. Stem Cells 26, 372–380.

Ocana, E., Carro-Carles, R., Sierra, P., Bajo-Muro, G., Acloque, H., Vega, S., Barral-Gimeno, A., Cano, A., Nieto, M.A., 2012. Metastasis colonization requires the repression of the epithelial–mesenchymal transition inducer Prrx1. Cancer Cell 22, 704–714.

Prince, M.E., Sivanandan, R., Kaczorowski, A., Wolf, G.T., Kaplan, M.J., Dalerba, P., Weissman, I.L., Wahl, G.M., 2006. Cancer stem cells...