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Title page

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Title: Phenotypic plasticity and epithelial-to-mesenchymal transition in the behaviour and therapeutic response of oral squamous cell carcinoma

Running title: EMT in oral SCC

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Keywords: EMT, MET, plasticity, heterogeneity, cancer

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Abstract

It is increasingly recognised that phenotypic plasticity, apparently driven by epigenetic mechanisms, plays a key role in tumour behaviour and markedly influences the important processes of therapeutic survival and metastasis. An important source of plasticity in malignancy is epithelial-to-mesenchymal transition (EMT), a common epigenetically-controlled event that results in transition of malignant cells between different phenotypic states that confer motility and enhance survival. In this review, we discuss the importance of phenotypic plasticity and its contribution to cellular heterogeneity in oral squamous cell carcinoma with emphasis on aspects of drug resistance and EMT.

Introduction

Carcinomas, malignant tumours of epithelial tissues, account for the great majority of all cancers and cancer-related deaths. The epithelial tissues in which cancers arise include barrier tissues such as the intestinal epithelium, skin and oral mucosa, as well as secretory structures such as the breast and prostate. In oral mucosa, the predominant tumour type is squamous cell carcinoma (SCC), which accounts for approximately 95% of all oral tumours and, unlike SCCs of the skin, are often highly locally invasive (1). Oral SCCs develop from cells in the basal layer of the oral epithelium and originate from either altered stem cells or through dedifferentiation of early-stage differentiated cells (2, 3). For metastatic dissemination to occur, malignant cells need to invade through both the basement membrane and the underlying connective tissue (3). Typically, oral SCC spreads by direct invasion or via the regional lymphatics to the cervical nodes of the neck, although haematogenous spread (particularly to the lungs) is possible. Metastasis to the neck, and beyond, is responsible for 90% of all oral SCC deaths (4). Late presentation, unpredictable patterns of metastasis, and limited therapeutic options for more advanced disease, put oral SCC in the top ten cancers worldwide by mortality rate (5).

A developmental basis for tumour heterogeneity

The term “epigenetics” describes heritable changes that occur in genetically identical cells and drive the diverse patterns of cell differentiation required to build all of the tissues of a complex organism from a single fertilized egg. Epigenetic mechanisms that elicit transcriptional changes include gene promoter methylation and histone modifications (particularly changes in histone acetylation) which alter accessibility of the DNA to RNAPol (6). Unlike permanent genetic changes, such epigenetic

changes are reversible and therefore enable a plasticity of cell phenotype. This plasticity was demonstrated half a century ago when it was shown by nuclear transfer experiments that the nucleus of a differentiated adult cell can substitute for the nucleus of a fertilized egg and drive the development of a new healthy adult (7). More recently, plasticity has also been demonstrated in cell culture, where it was found that addition of four transcription factors (Oct-4, Sox-2, c-myc and Klf4) was sufficient to convert a differentiated fibroblast into an embryonic stem cell that could give rise to all of the adult cell lineages (8). Transdifferentiation from one adult cell state to another has also been demonstrated, initially through cell fusion experiments using cells of two different lineages (9) but increasingly now through the addition of defined transcription factors (10). Although epigenetic states are not permanently fixed, epigenetic states imposed during development often persist in adult tissues to maintain the regionally-differing patterns of cell differentiation required for tissue renewal and function (Figure 1). This includes control of the hierarchies of stem and differentiating cells that underlie the continuous renewal of tissues such as the epidermis and oral mucosa (11). However, the potential plasticity of epigenetic states provides a mechanism for cells to adopt different phenotypic states and cell functions both during development and later in response to local signals (12). The normal diversity of epigenetic states in adult tissues has relevance for tumour development as tumours maintain, to a greater or lesser extent, the epigenetically-determined phenotypic states and patterns of differentiation seen in the parent tissue.

Tumour heterogeneity and therapy.

Successful treatment of oral SCC is challenging. In general, management of oral SCC is determined by factors that relate to the tumour (e.g. site, stage, bony invasion), the patient (physical status, preference) and available healthcare (expertise, cost, support). The three main treatment modalities for both curative and palliative management are surgery, radiotherapy and chemotherapy, which can be used in isolation or in combination with one another. Ionising radiation and chemotherapeutic agents, (usually platinum-based cisplatin, or combinations with 5-FU or newer taxanes) lead to DNA damage in dividing cancer cells, inducing cell death through apoptosis (13). Given its high success rate, primary radiotherapy may be the only treatment modality needed for small tumours, particularly those found in the soft palate, base of tongue and tonsillar region.

However, despite optimal surgical resection or primary radiotherapy, local recurrences and metastatic spread still occur. Indeed, incidences of systemic spread are increasing (14). Current standard radiation and surgical regimes may fail to eradicate all cancer cells as a few such cells may have invaded far beyond planned surgical and/or radiotherapy fields, and spread via the lymphatics or vasculature. There is a high likelihood that these disseminated cells might drive the growth of a

recurrent tumour, either at, or near, the primary site or at a distant secondary site (15). There is also evidence to suggest that such recurrent tumours are more aggressive compared to the initial, primary tumour, and that they display greater resistance to chemotherapeutic agents (16).

Resistance to chemotherapeutic agents can lead to treatment failure. Mutational changes occurring within a tumour can lead to increasing acquisition of therapeutic resistance by a process of clonal evolution (17). When subjected to a therapeutic challenge, cells within a tumour can undergo a form of Darwinian selection allowing genetic variants better able to withstand therapy to grow to become dominant populations. Epigenetic mechanisms can similarly provide cancer cells with differing resistances to therapeutic challenge (18). Unlike genetic changes, however, epigenetic changes can be driven by cellular signalling mechanisms derived from the tumour microenvironment through factors such as inflammation or hypoxia (19, 20). This provides a mechanism by which factors extrinsic to a tumour can influence its therapeutic responses. The flexibility of epigenetic changes means that, following a therapeutic insult, a small population of epigenetically-resistant cells can recapitulate a diversity of cell populations similar to those that comprised the original tumour. Such epigenetic changes can co-operate with genetic mechanisms to give a tumour time to acquire further resistance through mutation (21).

Aside from therapeutic resistance, another major question in cancer biology is that of invasion and metastasis. Malignant neoplasms range from well differentiated to undifferentiated, differentiation referring to the extent to which parenchymal cells resemble normal cells. Well-differentiated malignancies are less aggressive in nature, and less likely to metastasise, instead forming more discrete growths. Although they recognise no normal anatomic boundaries, their growth is more predictable and they are more amenable to surgical resection. Such tumours may have a good prognosis but many tumours, particularly those that are poorly- or un-differentiated, are much more likely to invade early into the surrounding tissue and metastasise via the lymphatic and circulatory systems. Complete surgical resection, where appropriate, is rendered more difficult, and prognosis is generally poorer compared to the well-differentiated malignancy. As is the case with therapeutic resistance, the ability to generate different epigenetic states is thought to be a key attribute of those tumours that can invade and metastasise (18). Employing this method, proliferating tumour cells can switch to a migratory phenotype allowing them to escape from the primary tumour and migrate to a distant site before then switching back to the initial proliferative phenotype to drive secondary tumour growth (15).

Two factors that must be delineated from each other are inter-tumoral and intra-tumoral genetic and epigenetic heterogeneity. Inter-tumoral heterogeneity is an important determinant of whether

a tumour has the ability to metastasise or resist therapy, and may underlie the large variation in progression and therapeutic response seen between different tumours. Intra-tumoral heterogeneity, by giving rise to a diversity of tumour cells with differing attributes within a single tumour, enables tumours to perform processes that confer therapeutic resistance and the ability to metastasise. Factors such as the initial genetic insults (a genetic difference) and the developmental state of the tumour cell of origin (an epigenetic difference) are likely to be of crucial importance to both types of heterogeneity. These two types of heterogeneity can be considered linked insofar as they both control what types of cells a particular tumour is capable of producing, and whether it contains cell types that are able to resist therapeutic interventions or invade and metastasise (Figure 2). It seems probable that both genetic and epigenetic factors cooperate to drive the processes that are observed in tumour development and progression.

Epithelial-to-mesenchymal transition, an important developmental process that drives tumour heterogeneity in oral squamous cell carcinoma.

One important epigenetic transition that provides a mechanism for both therapeutic survival and invasion/metastasis is epithelial-to-mesenchymal transition (EMT). During EMT, cells lose epithelial characteristics and gain mesenchymal ones, thereby enhancing their ability to migrate. This is achieved following a number of steps that lead to epithelial cells losing both their cell adhesiveness and apical-basal polarity, and undergoing both cytoskeletal and signalling changes which confer invasive properties.

EMT was first described as a process occurring during early embryonic development whereby ectodermal cells are enabled to migrate to the centre of the embryo to give rise to mesoderm (22). EMT also occurs later in development, initially for the specification of neural crest cells at the border between the developing neural tube and the rest of the ectoderm, and then to create specialist mesenchyme in various organs (23). During development, the reverse process of mesenchymal-to-epithelial transition (MET) also occurs, where populations of cells that have undergone EMT earlier in development return to an epithelial phenotype to give rise to secondary epithelial tissues such as the endocardium, epicardium, and urogenital precursors (23). These developmental processes point to a striking plasticity between epithelial and mesenchymal identities.

As well as at the earliest stages of life, EMT also occurs in inflammation in normal adult tissues. For example, during wound healing, epithelial cells at the wound edge undergo EMT which allows them to migrate directly into the wound. This normally follows exposure to a variety of stromal EMT-

inducing signalling factors from which they were previously isolated (24, 25). Tissue inflammation also aids EMT by facilitating recruitment of EMT-inducing immune cells to the wound site (26).

The process by which cells undergo a change from the epithelial phenotype to the mesenchymal is mediated by several stromal signals, including TGF β , I16 and TNF α , with TGF β currently the most studied. After TGF β binds to its cell receptors, the resulting nuclear translocation of Smad family transcription factors leads to interaction with transcription factors from the Snail, Twist and Zeb families, the EMT 'master regulators' (27-31). Other pathways downstream of TGF β that support EMT include phosphatidylinositol-3-kinase (PI3K) and mitogen-associated protein kinase (MAPK) (32). Together, their activity acts to repress those genes responsible for cell adhesion and polarity, such as RhoA and E-cadherin. This is supported by the loss of epithelial-specific intermediate filament keratin, and repression of claudin and occludin expression, all of which normally contribute to cell junctions. This repression is maintained through EMT (33). Additionally, N-cadherin is upregulated during EMT as E-cadherin is downregulated, the 'cadherin switch' (34). TGF β also mediates other properties of EMT cells, such as anti-apoptosis whereas in non-EMT cells it can have a pro-apoptotic role (35, 36). Other pathways involved in EMT involve hypoxia-inducible factor (HIF), which not only induces Twist and Snail expression, but also positively impacts upon pro-EMT β -catenin. β -catenin is normally kept at the plasma membrane of epithelial cells by E-cadherin (12) but HIF activity acts to support its translocation to the nucleus where it increases EMT-associated gene activity (37, 38). The Wnt signalling pathway plays a similar role in supporting the nuclear translocation of β -catenin (via inhibition of glycogen synthase kinase 3 β) and thus acts to support and maintain EMT (39). Receptor tyrosine kinase activation, by the binding of growth factors to the receptor tyrosine kinases, also induces EMT via up-regulation of Snail expression (40). Recent work has begun to explore the role of non-coding microRNAs, that selectively bind mRNAs and inhibit their translation, in modulating EMT. For example, the EMT-inhibitory miR-200 family of microRNAs represses translation of Zeb1 and Zeb2 mRNAs (41) and Zeb1 acts to suppress the miR-200 family (42). These mechanisms all support the loss of cell-cell attachments and the acquisition of a migratory mesenchymal phenotype.

As well as its physiological roles, EMT has now been shown to have several important roles in cancer (12), where EMT is promoted by both the inflammatory immune response (26, 43) and the hypoxic tumour environment (19, 44). A central role for EMT in the metastasis of several types of carcinoma, including oral SCC (44) and breast cancer (45), has been described. In some malignancies, cells appear to be primed to undergo EMT, possibly due to the possession of a range of relevant mutations (e.g. in receptor tyrosine kinase or Wnt signalling pathways) that predispose cells to undergo EMT (46).

Once cellular adhesion has been lost the resulting mesenchymal-like cells secrete matrix metalloproteinases (MMPs), allowing them to breach the basement membrane and invade into the tissue beyond (32). This is supported by cytoskeletal reorganization of the cell, itself enhanced by a key EMT protein, Vimentin, which allows the cell to develop cellular protrusions known as filopodia, lamellipodia and invadopodia (47, 48). These protrusions act to 'sense' the environment, support motility and secrete MMPs, leading to the behavior that typifies the EMT cell phenotype. The MMPs also act in a manner to support and maintain EMT in themselves, in a positive feedback mechanism (49).

A mouse xenograft study of oral SCC has shown that the hypoxia-inducible factor HIF1 α transcriptionally upregulates the EMT-inducing transcription factor Twist. This promotes EMT and metastasis of oral SCC, and expression of HIF1 α or Twist in primary human tumours can be correlated with metastasis and poor prognosis (44, 50). Other studies have demonstrated a correlation of upregulated Vimentin and downregulated E-cadherin with tumour invasion and metastasis (51-53). EMT not only facilitates movement but has also been implicated in therapeutic survival (54-56). Studies of both oral SCC and breast cancer have reported that EMT endows cancer cells with heightened resistance to chemotherapeutic agents and radiotherapy (54-56). Oral SCC cells that have undergone EMT have reduced oxygen consumption and greatly reduced production of reactive oxygen species (ROS), combined with upregulation of the ROS scavenger SOD2 (19). As reduced ROS and upregulated ROS scavengers have been shown to be associated with cancer radioresistance (16), this may form an important mechanism underlying the therapeutic resistance of cells that have undergone EMT.

The mechanisms inducing EMT have been extensively studied but, beyond the withdrawal of EMT inducing signals, there is little information about what induces the reverse process of MET. However, it is apparent that oral SCC cells are capable of entering two different states of EMT; one that is fixed and precludes return to an epithelial state, and another that is plastic and can undergo spontaneous MET to return to an epithelial phenotype (57, 58). As postulated by Brabletz (15), the combination of EMT and MET provides a mechanism for metastasis, as tumour cells can migrate away from the primary tumour and then undergo MET to enable new tumour growth (covered in detail in (59)). This concept is supported by findings that EMT can be detected at the invasive tumour edge and that secondary tumours typically re-express the same epithelial characteristics as the corresponding primary tumour (15). Direct evidence has now been provided by several recent studies (reviewed, (60)). One particularly interesting study (61) used a mouse model of metastatic skin SCC, in which tumours were induced by topical application of the chemical carcinogens DBMA and TPA. The mice

carried a doxycycline-inducible Twist-1 transgene, under the control of the Keratin 5 promoter, and this allowed selective induction of Twist expression leading to EMT in keratinocytes exposed to doxycycline. Systemic delivery of doxycycline induced Twist in all cancer cells, whereas local topical application of doxycycline was used to induce Twist only in the cells in the primary tumour. Systemic and topical doxycycline both increased the number of tumour cells entering the circulation and extravasating to the lung but only topical doxycycline resulted in an increased number of metastases, a difference interpreted as a requirement for reversal of EMT-associated growth arrest at the metastatic site and as evidence for sequential EMT and MET as drivers of metastasis in SCC (61).

Not all epigenetic mechanisms are of equivalent stability. In the case of fully repressed heterochromatin, genes are turned off in an essentially permanent fashion (with the exception of the special case of nuclear reprogramming to pluripotency, although even here there is great resistance to the reversal of heterochromatic states (62)). However, there is an epigenetic state termed "bivalent chromatin" that silences genes in such a way that the chromatin (DNA-histone complex) is still relatively open and genes are thus poised to be expressed when the correct combination of transcription factors is present. Bivalent chromatin was first discovered in embryonic stem cells where it enables many genes to remain poised to induce differentiation into a variety of lineages (63), but it is now known that key genes involved in EMT exist in a bivalent chromatin state in cancer cells; this facilitates phenotypic plasticity and enables cells to undergo EMT in response to various signalling cues (64). Further, it has been shown in breast cancer that cells that undergo EMT in response to stromal signals possess bivalent chromatin at the Zeb1 promoter, whereas those cells in which stromal signals cannot induce EMT possess a heterochromatic Zeb1 promoter (65). This provides a possible epigenetic mechanism for both inter-tumoral and intra-tumoral differences in the ability to undergo EMT.

Given the relative resistance to standard therapies of cells that have undergone EMT, it is now increasingly recognised that new ways of targeting these cells must be found. In malignant disease, the obvious aim is to impede or stop cells undergoing invasion and metastasis and the role of a number of drugs is currently being investigated. For example, after using E-cadherin shRNA to induce an EMT phenotype in breast cancer cells, the antibiotic Salinomycin was identified as an agent that could specifically target EMT cells (56). However, the mechanism by which it acts is yet to be elucidated. Another drug, Metformin, the oral anti-diabetic, has also been shown to target EMT cells in breast cancer (66) by inhibiting nuclear translocation of the EMT-inducing inflammatory transcription factor NF- κ B (67). The polyphenol Resveratrol has been shown to downregulate the key EMT master regulators Snail1, Snail2 and Zeb1 in mouse models of pancreatic cancer by repressing

their transcription (68). As pathways behind EMT are better understood, newer agents will be added to those currently being investigated.

Targeting signalling molecules that are important for maintenance of the EMT phenotype also provides a promising approach. One possible target is the cell surface glycoprotein CD44, which is involved in a variety of signalling pathways in cancer (69, 70) and is highly expressed on oral SCC cells that have undergone EMT (57). Due to alternative splicing of 9 variant exons, CD44 exists in many different isoforms that have different effects on cellular properties (70, 71). It has recently been demonstrated, for both oral SCC and breast cancer, that it is the standard isoform of CD44 containing no variant exons that is highly expressed on cells that have undergone EMT (71, 72). This might provide an opportunity for the development of anti-CD44 therapies that selectively target EMT cells.

Oral SCC is a potentially devastating disease, management of which often entails radical surgical intervention and extremely taxing chemo-radiotherapy regimes. Although survival and disease-free outcomes appear to have improved in recent years (73), the potential for significant further improvement exists. An extensive body of research now points to the key role of EMT in metastasis, in oral SCC and other cancers, and a better understanding of the complexities behind this process may offer the opportunity to modify, or develop new, chemotherapeutic agents which may serve to improve outcomes. Undoubtedly, new methods must be found that target EMT cells, or at least include them in therapeutic regimes, and a considerable amount of research effort is now being directed at this pressing issue.

Acknowledgements

This work was supported by the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs), by BAOMS/Saving Faces – FSRF, and by The Barts and The London Charity.

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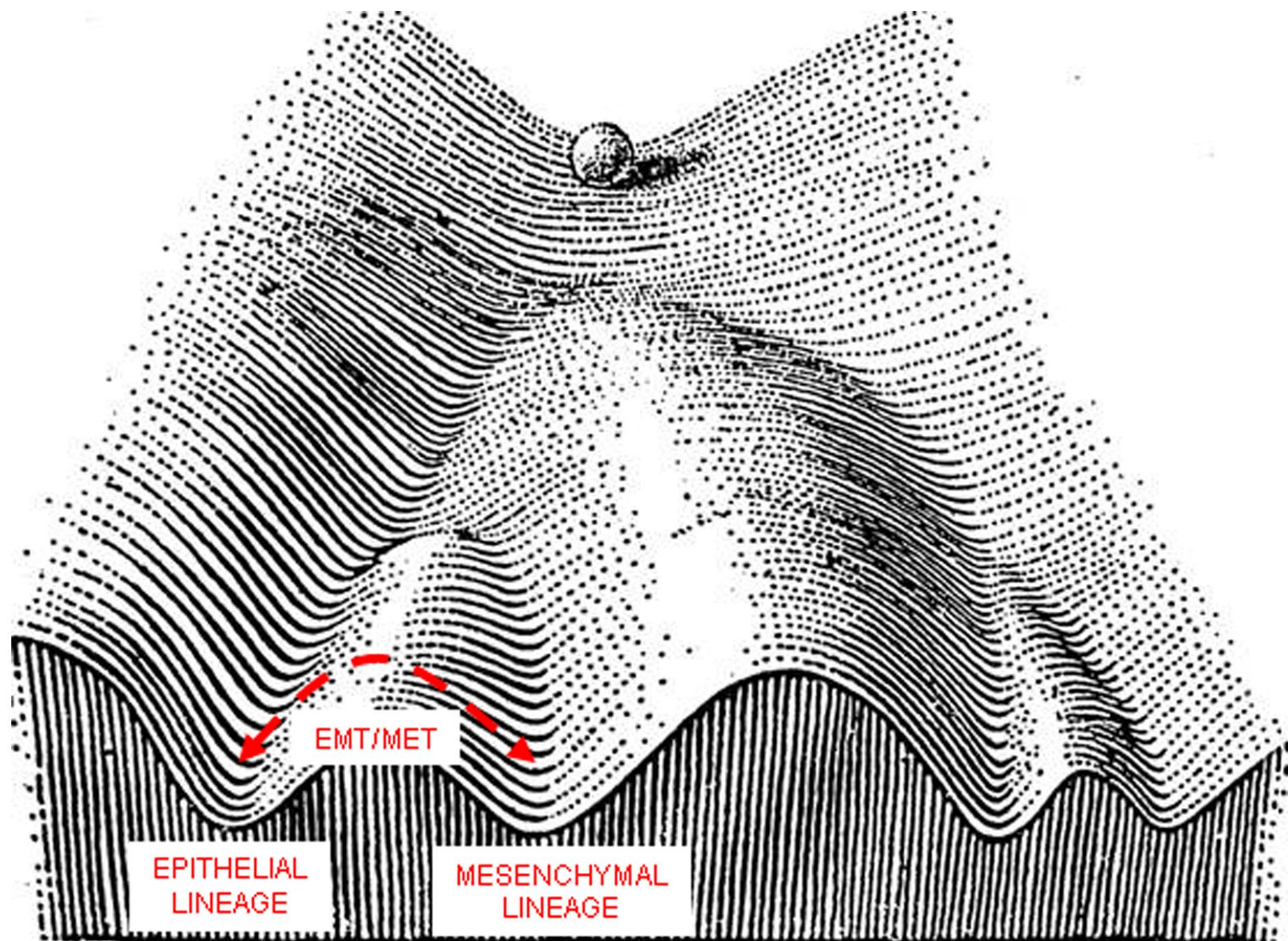
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Figure legends

Figure 1 – A limited level of epigenetic plasticity. This figure shows the ‘Epigenetic Landscape’ (Conrad Waddington, 1957) that has become the classic depiction of lineage restriction during development. The ball at the top of the hill represents a cell possessed of the totipotent state present in the fertilized egg (or the pluripotent state present in embryonic stem cells). As it rolls down the hill (representing fate choices during development) the cell must make choices between various valleys that restrict its future choice of identity. Epigenetic mechanisms close off genetic networks governing alternative lineages, leading to a restricted choice of cellular identity. However, it has recently become apparent that some cell types that were thought to be lineage-restricted (at the bottom of the hill) still possess a certain level of epigenetic plasticity, and one example of this is the ability to switch between epithelial and mesenchymal lineages by undergoing epithelial-to-mesenchymal transition (EMT) and mesenchymal-to-epithelial transition (MET). The poised state of ‘bivalent chromatin’ may be an important epigenetic state that enables this plasticity.

Figure 2 - Scheme illustrating the causes of inter-tumoral and intra-tumoral heterogeneity, and the consequences for therapy. In this scheme, two different epithelial cells of origin possess different epigenetic states and thus give rise to inter-tumoral heterogeneity. Cell of origin 1 (light blue) possesses a greater potential for phenotypic plasticity than cell of origin 2 (green), and thus requires fewer mutational events in order to facilitate epithelial-to-mesenchymal transition (EMT) (red cells). Differing mutational events in the cell of origin produce tumours with different genetic states, also contributing to inter-tumoral heterogeneity. Intra-tumoral heterogeneity in developing tumours can be driven by a) epigenetic changes (EMT, or differentiation into an alternative epithelial state), or b) mutational events in individual tumour cells to create genetically distinct clones within the tumour. Such events, creating intra-tumoral heterogeneity, also contribute to inter-tumoral heterogeneity and the breadth of differences in behaviour and therapeutic response between tumours. Intra-tumoral heterogeneity may enable a subset of tumour cells to survive a single type of therapy,

suggesting that it will likely be necessary to develop combinatorial therapies to ensure that all tumour cells are targeted.



Outcomes of therapeutic interventions using 'Drug A' and 'Drug B'

Inter-tumoral genetic and epigenetic heterogeneity

