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1 **CELL ADHESION AND UROTHELIAL BLADDER CANCER:**
2 **THE ROLE OF CADHERIN SWITCHING AND RELATED PHENOMENA**

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24 **ABSTRACT**

25 Cadherins are mediators of cell-cell adhesion in epithelial tissues. E-cadherin is a known tumour suppressor and
26 plays a central role in suppressing the invasive phenotype of cancer cells. However, the abnormal expression of N-
27 and P-cadherin (“cadherin switching”) has been shown to promote a more invasive and malignant phenotype of
28 cancer, with P-cadherin possibly acting as a key mediator of invasion and metastasis in bladder cancer. Cadherins are
29 also implicated in numerous signalling events related to embryonic development, tissue morphogenesis, and
30 homeostasis. It is these wide-ranging effects and the serious implications of cadherin switching that make the
31 cadherin cell adhesion molecules and their related pathways strong candidate targets for the inhibition of cancer
32 progression, including bladder cancer. This review will focus on cadherin switching in the context of bladder cancer
33 and in particular the switch to P-cadherin expression, and will discuss other related molecules and phenomena,
34 including EpCAM and the development of the cancer stem cell phenotype.

35

36 **MEDIA SUMMARY**

37 Cadherins are mediators of cell-cell adhesion in epithelial tissues. E-cadherin is a tumour suppressor and plays a
38 central role in suppressing the invasive phenotype of cancer cells. However, the abnormal expression of other
39 cadherins (“cadherin switching”) has been shown to promote a more invasive and malignant phenotype of cancer.
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41 morphogenesis, and homeostasis. It is these wide-ranging effects and the serious implications of cadherin switching
42 that make the cadherin cell adhesion molecules and related pathways attractive targets for the inhibition of cancer
43 progression, including bladder cancer.

44 **BLADDER CANCER**

45 **Introduction**

46 Urothelial bladder cancer (UBC) is the fifth most common cancer in Western society, with a global incidence of over
47 356,000 and a prevalence estimated at 2.7million [1;2]. The burden of the disease is predicted to increase
48 significantly in the foreseeable future as a result of population aging and the increasing world population, together
49 with the progression of the tobacco epidemic and increasing exposure to occupational carcinogens in developing
50 countries [2]. In the UK there are approximately 10,200 new cases and 5,000 deaths attributed to bladder cancer per
51 year [3]. In Western populations over 90% of bladder cancers are transitional cell carcinomas of urothelial origin
52 (urothelial cancers, UCs), and at presentation 75-85% will be non-muscle-invasive tumours (NMIBC, stages
53 Ta/T1/Tis), with the remainder being muscle-invasive (MIBC, stages T2-4) [1;4-6].

54 NMIBC is a heterogeneous disease typified by a high rate of recurrence (15-61% at one year, depending upon risk
55 category [7]) and so long-term, even lifelong, surveillance with outpatient flexible cystoscopy is the mainstay of
56 subsequent management [6;8]. Progression to MIBC is also a concern for high-risk NMIBC patients, occurring in up to
57 17% of patients at one year [7]. However, the overall prognosis is good with 65-85% of patients surviving for 5 years
58 or more [5].

59 Progression to (or presentation with) MIBC represents the critical step in the disease course, necessitating more
60 radical therapies and carrying a 5-year survival rate of only 25-50% [5;9]. For curative intent, patients who present
61 with or progress to MIBC are treated by radiotherapy [6;10], chemoradiotherapy [11], radical cystectomy, or
62 neoadjuvant chemotherapy followed by radical cystectomy [6;9;10].

63 The cumulative cost of treating UBC exceeds all other forms of human cancer, the majority of which is attributable to
64 the long-term treatment and surveillance of NMIBC [12-14]. Despite this, there is only modest research funding for
65 UBC compared to other malignancies [15], and as a result there has been a lack of scientific advancement in the field
66 [15-17], with no major new drugs approved for UBC in over 10-years [17;18].

67 Cadherins are mediators of cell-cell adhesion in epithelial tissues [19;20]. We have previously demonstrated that the
68 abnormal expression of P-cadherin (an example of "cadherin switching") is associated with an invasive and

69 aggressive phenotype of UBC [21], and have hypothesized that P-cadherin may act as a key effector of muscle-
70 invasion [22]. The cadherins are involved in a number of important phenomena related to cancer progression,
71 including epithelial-to-mesenchymal transition (EMT) and the development of a cancer stem cell phenotype [22;23].
72 It is these wide-ranging effects and the serious implications of cadherin switching that make the cadherins and their
73 related pathways strong candidate targets for the inhibition of cancer progression, including UBC. This review will
74 focus on cadherin-based cell adhesion in the context of UBC and the switch to P-cadherin expression, and will discuss
75 other related molecules and phenomena, including EpCAM and the development of the cancer stem cell phenotype.

77 **METHODS**

78 Our group has been working in the field of cadherin biology for a number of years [24;25], and we regularly review
79 the literature on these molecules and their associated pathways [22]. Specifically, this review was written utilising
80 papers obtained following *PubMed* searches and with the following structure: bladder cancer background,
81 epidemiology and molecular pathogenesis; cadherin background and biology; cadherins in epithelial malignancies,
82 cadherin switching, and cadherins in bladder cancer. The background to cadherins and cadherin biology presented
83 here has been derived from key papers by workers who initially characterised and described these molecules, and
84 then who subsequently investigated cadherin expression and function in various epithelial malignancies and model
85 systems. We updated the field for cadherin switching to describe this process in the context of malignancy and
86 related phenomena (eg. epithelial-to-mesenchymal transition, cell migration, metastasis, cancer stem cells, EpCAM
87 signalling), utilising papers written by significant workers in this field. The data, findings and information contained
88 within these publications were then assimilated to create a review of cadherin switching in bladder cancer and
89 including some of our own interpretations.

91 **MOLECULAR PATHWAYS TO NON-MUSCLE-INVASIVE & MUSCLE-INVASIVE BLADDER CANCER**

92 Different approaches have been taken to describe the molecular alterations involved in bladder tumorigenesis [26-
93 31]. We have previously described such pathways based upon the six “hallmarks of cancer” described by Hanahan

94 and Weinberg in 2000 [32-35]. In 2011 Hanahan and Weinberg updated their original landmark review, describing
95 genome instability and inflammation as underlying these hallmark changes, and proposed “reprogramming of
96 energy metabolism” and “evading immune destruction” as two emerging hallmarks with potential for generality
97 [35]. In addition, they described that tumors exhibit another dimension of complexity by containing a repertoire of
98 recruited, ostensibly normal cells that contribute to the acquisition of hallmark traits by creating the "tumor
99 microenvironment" [35], and our own research has demonstrated the apparent importance of the immunological
100 milieu of the bladder tumour microenvironment [RT Bryan et al - unpublished data]. In their 2011 update, Hanahan
101 and Weinberg also introduced the concept of “cancer stem cells” [35], a concept that has existed for a number of
102 years in haematopoietic malignancies [36;37]. Cancer stem cells (CSCs) are a subset of tumor cells that have the
103 ability to self-renew and to generate all of the heterogeneous cells that comprise a tumor (properties that are
104 analogous to a stem cell, the original cell of an organ and responsible for organogenesis and organ maintenance)
105 [23;36;38-40]. In the setting of UBC, CSCs appear to play a role in a subset of tumors, but their true significance is yet
106 to be clarified [23].

107 Other authors have reviewed the field of UBC molecular pathogenesis in detail [26-31], and there has been general
108 consensus on a divergent pathway for the development of Ta/T1 disease and Tis/T2+ disease [29;41-46]. However,
109 Dancik et al recently identified a cell of origin gene signature for basal cells and umbrella cells of the urothelium [47].
110 By utilising this cell of origin signature in UBCs from 874 patients, it appeared that NMIBCs and MIBCs developed
111 from distinct progenitor cells [47], possibly shifting our understanding of urothelial carcinogenesis away from the
112 classical two pathway model. Further detailed genomic and epigenomic studies of both MIBCs and NMIBCs are thus
113 required to clarify our understanding of the pathogenesis of these tumours [48].

114 Although a detailed examination of these pathways is beyond the scope of this review, this is a rapidly changing field
115 and new developments appear frequently with the advent of high-throughput experimental platforms including
116 “deep sequencing” [49], proteomics [50-52] and metabolomics [53]. Most recently, The Cancer Genome Atlas (TCGA)
117 Research Network undertook the comprehensive molecular characterization of 131 MIBCs [49]. With regard to
118 somatic DNA mutations, a notable finding was the significant enrichment of non-silent mutations in chromatin
119 regulatory genes compared to other epithelial cancers studied: 76% of the tumours (MIBCs) had an inactivating

120 mutation in one or more of these genes, and 41% had at least two such mutations [49]. *TP53* mutations were also
121 common (49%), as were amplification and overexpression of *MDM2*, suggesting that TP53 function was inactivated
122 in 76% of tumours [49]. There were a large number of previously undescribed mutations, and viral DNAs and
123 transcripts were also indentified [49]. RNA-seq data identified 4 tumour clusters and pathway analysis demonstrated
124 three frequently dysregulated pathways [49]: cell-cycle regulation (altered in 93% of cases); kinase and
125 phosphatidylinositol-3-OH kinase (PI(3)K) signaling (72%); and chromatin remodelling (89%). A number of the
126 genomic alterations indentified are theoretically amenable to therapeutic targeting [49], and such new therapeutics
127 are desperately needed for UBC [17;18;54].

128 Choi et al also utilised whole genome mRNA expression profiling to cluster MIBCs into 3 distinct groups, based upon
129 the established molecular subtypes of breast cancer [55]: basal MIBCs shared biomarkers with basal breast cancers
130 and were characterized by p63 activation, squamous differentiation, and more aggressive disease; luminal MIBCs
131 contained features of active PPAR γ and oestrogen receptor transcription and were enriched with activating *FGFR3*
132 mutations and potential *FGFR* inhibitor sensitivity; p53-like MIBCs were consistently resistant to a number of
133 chemotherapeutics, including cisplatin; and all chemoresistant tumours adopted a p53-like phenotype after therapy
134 [55]. These findings have important implications for the clinical management of MIBC: they include not only
135 prognostic information, but also suggestions for subtype-directed targeted therapy and potential to predict response
136 to cisplatin-based chemotherapy (although further work is needed to elucidate other biomarkers of resistance) [56].
137 It is, however, disappointing that NMIBCs were not analysed in the same way by either the TCGA Research Network
138 or Choi et al [48], especially as these tumours represent the vast majority (>75%) of bladder cancer patients [57;58].

140 **CADHERINS**

141 The classical cadherins are calcium-dependent transmembrane glycoproteins found at the adherens junction and are
142 mediators of cell-cell adhesion in epithelial tissues [19;20]. E-cadherin is a tumour suppressor, playing a central role
143 in suppressing the invasive phenotype of UBC cells [59]. The abnormal expression of other “classical” cadherins (P-
144 and N-cadherin) has been shown to promote a more invasive and malignant phenotype of UBC [24], possibly acting

145 as key mediators of invasion and metastasis. With such a large difference in UBC outcomes between early stage
146 disease (stage Ta) versus MIBC (stages T2+) it is reasonable to assume that cell adhesion molecules, and in particular
147 cadherins, play a fundamental role in the spread of bladder tumours, initially from the urothelium into the lamina
148 propria (through the basement membrane) and subsequently into the detrusor muscle [22]. Therefore, the classical
149 cadherins and their related molecular pathways represent attractive therapeutic targets for the inhibition of
150 progression in bladder cancer patients [19;59-61].

151 Cadherins comprise of extracellular (EC1-5), transmembranous, and cytoplasmic domains, with the cytoplasmic
152 domain anchored to the cell cytoskeleton by catenin family members (α -, β -, γ -catenin and p120) [19;61-65]. P21-
153 activated kinase 5 (PAK5) also appears to associate with β -catenin and p120 to stabilise the adherens junction in
154 order to maintain normal cell-cell adhesion [66]. Traditionally, cell-cell adhesion is described as being achieved by
155 the symmetric interactions of the first extracellular domains (EC1) of cadherins on neighbouring cells (trans-
156 interaction) [64;67]; cadherins on the same cell also interact with each other (cis-interaction) through the EC1
157 domain of one and the EC2 domain of the other [64;67;68]. More recently, it has been described that optimal cell-
158 cell adhesion (50-70pN) is achieved by all 5 EC domains of E-cadherin, and with a cell-cell separation of 5-11nm [65].
159 See **Figure 1**. E-, P- and N-cadherin were the first cadherins identified, and can all mediate cell-cell adhesion in this
160 fashion [63;69]:

- 161 • E-cadherin (CDH1, 120kDa): the main mediator of cell-cell adhesion in epithelial tissues and expressed by
162 most normal epithelial cells [19;61;62;69-71].
- 163 • N-cadherin (CDH2, 130kDa): expressed by neural, endothelial, and muscle cells, but not normally by
164 epithelial cells [62;69].
- 165 • P-cadherin (CDH3, 118kDa): normally only weakly expressed in the basal layers of stratified epithelia such as
166 oesophagus, bronchus and bladder [24;69;71].

167 Epithelial malignancies, including bladder cancer, typically show loss of E-cadherin expression as grade and stage
168 progress, and this is often accompanied by increased expression of N- or P-cadherin. This phenomenon is described
169 as “cadherin switching” [19;61;69;71-73], illustrated in the bladder cancer setting in **Figure 2**. Excellent reviews of

170 the field have been published recently [74;75], and we have previously reviewed this field for bladder cancer [22];
171 we provide an overview below.

172

173 **CADHERIN SWITCHING**

174 Cadherin switching (CS) is a hallmark of epithelial-to-mesenchymal transition (EMT) [76], the process by which
175 epithelial cells lose their characteristic polarity, disassemble cell junctions, and become more migratory as a
176 precursor to invasion and metastasis (they acquire properties analogous to mesenchymal cells) [19;25;61;77-82]. In
177 this setting, CS typically describes a process where the normal expression of E-cadherin is replaced by the abnormal
178 expression of N-cadherin, or where N-cadherin expression is increased and E-cadherin levels remain unchanged
179 [19;61;76]. CS appears to play a role late in many malignancies (including breast, prostate, pancreas, ovarian,
180 bladder and melanoma), resulting in a more invasive and malignant phenotype of disease with a worse outcome
181 [19;24;61;74-76;83-89]. The regulation of CS is yet to be fully elucidated, but most likely involves transcriptional and
182 post-transcriptional events, possibly influenced by cytokines or growth factors [19;61]. Recently, Slug (*SNAI2*, a
183 member of the Snail family of zinc-finger transcription factors) has been identified to play a critical role in EMT by
184 control of the E-cadherin to N-cadherin switch in UBC [90].

185 In UBC, ourselves and others have described CS, demonstrating increased expression of both P- and N-cadherin in
186 late stage high-grade disease (**Figure 2**) [24;69;89;91;92]. We studied 153 bladder tumours and utilised a variety of
187 cell lines and functional in vitro models [24]: increased membranous P-cadherin expression was observed in almost
188 half of all MIBCs and almost 40% of grade 3 UBCs, accompanied by significantly reduced expression of E-cadherin
189 [24]. Increased P-cadherin expression was associated with worse bladder cancer-specific survival, and P-cadherin
190 status was an independent prognostic factor (alongside grade and stage) [24]. Functional in vitro experiments
191 showed that altering the balance of E- and P-cadherin in favour of P-cadherin expression enhanced anchorage-
192 independent growth, and that P-cadherin alone was unable to mediate normal cell-cell adhesion [24]. We concluded
193 that P-cadherin expression promoted a more malignant and invasive phenotype of bladder cancer (even in the
194 presence of E-cadherin), and appeared to have a novel role late in the disease process [24].

195 Mandeville et al also demonstrated similar findings [92]. In their in vitro studies, utilising P-cadherin transfection and
196 knockdown, they demonstrated that P-cadherin induced a significant increase in migratory capacity (although with
197 no accompanying change in invasive potential) [92]. The authors suggested that P-cadherin may have a role in
198 regulating the migration of basal cells to the intermediate cell layer in normal urothelium, as well as a role in
199 neoplastic progression [92]. More recently, Wang et al have demonstrated similar findings [89].

200 Ourselves and others have postulated that a subgroup of aggressive P-cadherin-expressing tumours may be derived
201 from the normally weakly P-cadherin-expressing basal layer of the urothelium [22]. In support of this hypothesis,
202 Van Batavia et al recently demonstrated that papillary and CIS lesions were derived from different urothelial
203 populations, with intermediate cells contributing to non-invasive papillary lesions and basal cells representing the
204 origin of CIS (which ultimately leads to MIBC) [93]. These findings support a model in which the heterogeneity
205 observed in bladder cancers is determined both by genetic changes and the cell lineage from which the tumour
206 originates [93].

207 However, despite P-cadherin expression being associated with a more aggressive phenotype in many cancers, such
208 behaviour is not ubiquitous and is context dependent [75]. For example, in malignant melanoma, which commonly
209 demonstrates a cadherin switch to N-cadherin expression [22], P-cadherin promotes adhesion and inhibits invasion
210 in a similar fashion to E-cadherin [75], and E-cadherin negative breast cancer cells show many similarities when
211 subsequently transfected with E- or P-cadherin [74;94]. Ribeiro et al investigated these phenomena in detail in a
212 breast cancer model, demonstrating that P-cadherin co-localizes with E-cadherin, and promotes cell invasion by
213 disrupting E-cadherin/catenin interactions [95]. E- and P-cadherin co-expressing tumour cells showed enhanced in
214 vivo tumour growth compared with those expressing only E- or only P-cadherin, and co-expression of E- and P-
215 cadherin in breast tumours correlated with high-grade biologically aggressive tumours accompanied by poor patient
216 survival [95]. It is therefore feasible that P-cadherin only promotes invasion in tissues that endogenously express E-
217 cadherin [74], with heterodimerisation between E- and P-cadherin disrupting the formation of functional cadherin-
218 catenin complexes [75].

219 It is likely that the key mechanisms involved in P-cadherin's deregulation largely occur in the promoter region of
220 *CDH3* and not by structural alterations of its coding sequences [74]: in 2005, Paredes et al demonstrated

221 hypomethylation of the *CDH3* gene promoter correlated with P-cadherin overexpression in breast cancer [74;96],
222 and other workers have described this phenomenon in pancreatic [74;97] and colorectal cancers [74;98]. Our own
223 data suggest differential *CDH3* promoter methylation between bladder cancer cell lines and tumours, and normal
224 urothelium [RT Bryan - unpublished data]. Furthermore, the balance of E- and P-cadherin expression impacts the
225 overall genetic programme [74], altering the expression of genes involved in signal transduction and growth factors,
226 cell cycle, cell adhesion and the extracellular matrix, cytokines and inflammation [74;94]. In addition, P-cadherin can
227 provoke the secretion of pro-invasive factors such as the matrix metalloproteinases MMP1 and MMP2 [74;75;99].
228 The role of p120 also appears important, with P-cadherin probably interfering with the normal binding of p120 to E-
229 cadherin at the adherens junction [74;100]. In a pancreatic cancer model, accumulation of p120 in the cytoplasm
230 (and not bound to E-cadherin at the membrane) appeared to induce the increased cell migration seen following P-
231 cadherin expression via the Rho GTPases, Rac1 and Cdc42 [74;101]. P-cadherin-induced increase in Rac1 and Cdc42
232 activity (mediated via p120) has also been observed in ovarian cancer [74;102]. Specifically, insulin-like growth factor
233 1 receptor (IGF1R) can seemingly form a complex with P-cadherin, resulting in the tyrosine phosphorylation and
234 activation of cytoplasmic p120 to promote invasion [75;102;103]; this pathway appears specific to P-cadherin and
235 not the other classical cadherins [75;103].

236 Taken together, all of the data above emphasise that P-cadherin represents a very attractive target for novel anti-
237 cancer therapeutics [74], and phase I trials of a P-cadherin inhibitor (PF-03732010, a human monoclonal antibody
238 against P-cadherin) have been undertaken [104], although its development now seems to have stalled.

239

240 **CADHERINS AND CANCER STEM CELLS**

241 Although solid tumours can be reduced in size or eradicated by chemotherapy, radiotherapy or surgery (alone or in
242 combinations), disease relapse or progression often occurs [105;106]. Such relapse or progression may be explained
243 by the persistence of residual tumour-initiating cells and tumour-maintaining cells, and such cells have been
244 reported in a variety of malignancies (breast, brain, prostate, lung, pancreas, etc) since they were first identified in
245 leukaemia [79;105;107]. Such “cancer stem cells” (CSCs) theoretically have the ability to self-renew and to generate

246 the heterogeneous cells that comprise a tumour [105-110], and thus need to be eradicated to provide long-term
247 disease-free survival (although it appears that CSCs are more resistant to conventional therapies) [108;110-112].
248 CSCs may either develop following genetic or epigenetic events in normal stem cells or from differentiated tumour
249 cells that develop the capability for unlimited growth [23;82]. Cellular markers of “stemness” are still under debate,
250 but include CD44, CD24, CD133 and EpCAM [82]: in breast, prostate and oral squamous carcinomas, CSCs are likely
251 identified as CD44⁺/CD24⁻, whereas CD133 appears to be a CSC marker in gliomas and in colon and pancreatic
252 carcinomas [82].

253 In a previous review we suggested that the evidence supports the CSC paradigm for UBC, as in other epithelial
254 malignancies [23]. As discussed above, in normal urothelium P-cadherin is only expressed in the basal cell layer (the
255 assumed urothelial stem cell niche) and in a subset of more aggressive UBCs [21-23;92;113]. It is therefore tempting
256 to assume that P-cadherin is a marker of urothelial stem cells and UBC CSCs. Although E-cadherin intercellular
257 adhesion is considered important for the survival of human embryonic stem cells (hESCs) and induced pluripotent
258 stem cells (iPSCs) [82], Kolle et al recently identified *CDH3* (P-cadherin) and *TACSTD1* (EpCAM) as genes encoding
259 hESC markers (antibodies for EpCAM were also able to enrich for pluripotent hESCs) [114]. Vieira et al have also
260 demonstrated that P-cadherin mediates stem cell properties in basal-like breast cancer [115]. P-cadherin therefore
261 appears promising as a potential marker of CSCs in UBC, and similar work is required to confirm these findings in
262 UBC [23]. The fact that *CDH3* (P-cadherin) did not appear in Dancik et al’s cell of origin signature described earlier is
263 somewhat surprising since it is normally expressed by basal urothelial cells and in a subset of aggressive UBCs that
264 may also harbour CSCs; however, as described above, P-cadherin’s deregulation is most likely governed by
265 epigenetic phenomena rather than structural alterations in its coding sequences [74]. Characterisation of the UBC
266 epigenome/methylome may thus be required to elucidate P-cadherin’s role in these UBC subtypes.

267 It is highly feasible that treatment-resistant cells develop via other mechanisms and pathways, with CSCs being
268 responsible only for a minority [105;116]. Heterogeneity within some tumours may result from selective pressure
269 during tumorigenesis [35;112]. See **Figure 3**. It has been suggested that UBCs arise from more differentiated cells,
270 and self-renewal capacity may be acquired secondarily by inactivation of *p53* and *RB1* function [105;116]. The
271 tumour microenvironment may also play an important role [108], potentially inducing a transitory or reversible CSC-

272 like state [117]: although EMT may drive the development of CSCs [35], EMT itself is reversible with mesenchymal-
273 to-epithelial transition (MET) favouring a cell's colonisation of distant sites to generate metastases [35]. Whether the
274 CSC state reverses in a similar setting and fashion remains unknown, but such interactions highlight the importance
275 of the tumour microenvironment for all cancer cells, not just CSCs [35].

277 CADHERINS AND EPCAM

278 EpCAM is a type-1 membrane protein that functions as a cell adhesion molecule [118]. It is overexpressed in many
279 epithelial malignancies, including bladder CIS [119] and high grade and advanced stage UBCs [120]. The tumour-
280 specific expression of EpCAM has led to its use for capturing circulating tumour cells by the FDA-approved
281 *CELLSEARCH* system [121], and also for directing therapies to bladder tumours [122]. High tissue levels of EpCAM are
282 associated with a poor prognosis in UBC [120]. However, the role of EpCAM remains elusive: both tumour
283 suppressor and oncogenic properties have been reported. In 2009, Maetzel et al demonstrated that EpCAM could be
284 sequentially cleaved to release extracellular and intracellular domains, 'EpEX' and 'EpICD', respectively [123]; EpICD
285 diffuses into the nucleus and activates oncogenic signalling events by associating with FHL2, β -catenin and Lef-1
286 [123;124]. See **Figure 4**.

287 In 2014, as part of our de novo urinary biomarker discovery programme [125], we demonstrated that elevated
288 urinary EpCAM was observed in patients with grade 3 NMIBCs and MIBCs [51;52]. EpCAM was a significant
289 independent prognostic factor for UBC-specific survival, with elevated urinary levels resulting in an increased risk of
290 dying from bladder cancer (hazard ratio 1.76). The predominant form of EpCAM in the urine was a soluble and stable
291 form comprised of the entire extracellular domain, and not the intact protein [52]. Our data therefore suggested
292 that the cleavage of EpCAM into EpEX and EpICD could also occur in UBC [52;123], and further evidence supports
293 this: Ralhan *et al* recently demonstrated that 9 out of 10 cases of UBC were positive for EpICD [126]. However, our
294 work demonstrated that the extracellular domain of EpCAM was released by cleavage immediately adjacent to the
295 cell membrane [52]; the exact location of cleavage was not described by Maetzel et al [123], but the protease

296 involved (TACE or ADAM 17) usually cleaves membrane proteins 10-15 residues away from the membrane surface
297 [127], suggesting atypical cleavage or an alternative mechanism of extracellular domain release in UBC [52].

298 Notably, there are important relationships between EpCAM and classical cadherins, although this relationship
299 appears to be tissue- and tumour-specific [128]. In 1997, Litvinov et al suggested that EpCAM has a role in the
300 development of a proliferative and malignant phenotype of epithelial cell [129]: increasing the expression of EpCAM
301 in cadherin-positive cells led to the gradual abrogation of adherens junctions [129]. Although EpCAM had no
302 influence on the total amount of cellular cadherin, it affected the interaction of the cadherins with the cytoskeleton
303 and, as cadherin-mediated cell-cell adhesion diminished, EpCAM-mediated intercellular connections predominated
304 [129]. In a murine fibroblast model, Winter et al subsequently demonstrated that this may occur by disruption of the
305 link between α -catenin and F-actin, probably by EpCAM's disruption of the actin cytoskeleton or possibly via p120
306 [130]. In later work on human breast epithelial cells, the same authors demonstrated that EpCAM cross-signaling
307 with N-cadherin resulted in the abrogation of cadherin adhesion complexes, mediated by PI(3)K [131]. In breast
308 cancer cell lines, Martowicz et al showed that epithelial cells need EpCAM to promote growth and invasion, yet
309 mesenchymal tumour cells are independent of EpCAM for invasion and progression [132]; the same authors also
310 demonstrated that overexpression of EpCAM in human mammary epithelial cells led to a more proliferative
311 phenotype and downregulation of E-cadherin [133].

312 Conversely, in a zebrafish model, Slanchev et al demonstrated that EpCAM was indispensable for skin epithelial
313 integrity, and that *epcam* mutant embryos displayed reduced levels of membranous E-cadherin [134]. Guerra et al
314 also postulated an important role for EpCAM in the maintenance of normal intestinal architecture and function in
315 congenital tufting enteropathy, utilising an *mTrop1/Epcam* knockout mouse model of the disease [135]. Other model
316 systems have also demonstrated a direct association between loss of EpCAM expression and loss of cadherin-
317 mediated adhesion [136].

318 Seemingly, EpCAM has dual functions in normal and cancerous cells with regard to cadherin regulation, cell-cell
319 adhesion and epithelial integrity: EpCAM may be essential for normal epithelial tissue integrity and cell-cell
320 adhesion, but there also appears to be a role for EpCAM in the disruption of normal cell-cell adhesion to initiate
321 EMT, with the subsequent transformed cells acting independently of EpCAM signaling for invasion and progression.

322 Interestingly, Zeb1 (a known transcription factor inducing EMT) represses both E-cadherin and EpCAM by binding to
323 the EpCAM promoter [137], yet the expression of E-cadherin and EpCAM is related to a stem cell-like phenotype
324 [138;139]; in basal-like breast cancer EpCAM and P-cadherin both appear to be associated with the CSC phenotype
325 [115]. As described for the hallmarks of cancer [34], the timing and ordering of these events appears to differ
326 between normal and tumorous tissues, between different tissue and tumour types, and most likely within the same
327 tumour. It is feasible that during EMT in some malignancies, EpCAM may stimulate the dissolution of E-
328 cadherin/catenin complexes and so permit P- and N-cadherin complexes to predominate (cadherin switching) and β -
329 catenin-mediated oncogene transcription to be upregulated; yet in other tumour types, EpCAM and E-cadherin may
330 be downregulated in parallel, with EMT being driven by alternate pathways. Conversely, EpCAM may stabilise E-
331 cadherin/catenin complexes in some tumours, possibly providing a "stable" and less chaotic cellular milieu
332 unaffected by EMT, in which the development of a CSC phenotype can be "nurtured" by alternative pathways (as
333 described above, EpCAM is a cell surface marker of hESCs, and can be used to isolate a pluripotent subpopulation
334 from hESC culture [114]). If the latter model is correct, then the corollary would potentially be the normalisation of
335 β -catenin-mediated transcription in CSCs; evidence to date in other malignancies suggests that this is not the case
336 [140-142]. However, these are dynamic processes, and even within the same tumour all of these proposed
337 phenomena may be unfolding simultaneously; in the future, single cell genomics may resolve these issues [143;144].
338 It is important to note that CSC-like treatment-resistant disease may develop via alternate pathways (Figure 3), and
339 there is likely to be considerable plasticity [142], with cells reverting to a less aggressive state by mesenchymal-to-
340 epithelial transition (MET) or by the reversal of the CSC phenotype. Furthermore, the influence of EpCAM on P-
341 cadherin is yet to be elucidated. Our current research is attempting to resolve some of these mechanisms.

344 **DISCUSSION & CONCLUSION**

345 P-cadherin seemingly has a number of fundamental roles in bladder cancer and other malignancies, including
346 mediating the development of CSCs and EMT, both of which lead to more aggressive disease and worse survival. The
347 mechanisms of these phenomena have been well-described in other malignancies, but remain to be elucidated in
348 UBC. Although we have assumed some crossover of P-cadherin's function between tumour and tissue types, we
349 know that many of P-cadherin's actions are tumour- and tissue-specific. Therefore, such findings from other
350 malignancies need to be reproduced in UBC if we are to genuinely understand P-cadherin's role in this setting.
351 However, given the genomic characterizations of MIBC described above [47;49;55], it is unlikely that P-cadherin
352 represents a "driver" of urothelial carcinogenesis [145]; P-cadherin is more likely to represent an important
353 downstream effector of such driver mutations, with multiple influences on important pathways and phenomena that
354 determine outcomes in advanced disease (eg. EMT, CSCs), probably mediated by PI(3)K [49]. Moreover, it appears
355 that P-cadherin plays a fundamental role in the cell surface and cell adhesion phenomena that permit tumour cells
356 to migrate and invade, and possibly to metastasize.

357 In conclusion, P-cadherin represents a highly attractive therapeutic target, alongside N-cadherin [146-148]. However,
358 given P-cadherin's complex interactions described above (and undoubtedly many yet to be discovered), P-cadherin
359 inhibition may have far more wide-reaching effects than those directly related to tumour invasion and progression.
360 The difficulties of taking an anti-P-cadherin agent through clinical trials and into clinical use should therefore not be
361 underestimated. Furthermore, the association of classical cadherins with EpCAM is particularly fascinating and
362 requires further elucidation in UBC, and our work in this area is ongoing.

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706 LEGENDS FOR FIGURES

707 **Figure 1: Cell-cell adhesion in epithelial tissues** (taken from [22]). **a)** overview of cell-cell adhesion complexes; **b)**
708 pictorial representation of cell-cell interactions on neighbouring cells; **c)** molecular structure of the adherens
709 junction, showing the relationship between E-cadherin molecules on neighbouring cells, and between E-cadherin,
710 the catenins (α , β , γ , p120) and the cell cytoskeleton. Traditionally, cadherins on neighbouring cells adhere via EC1
711 domains, although more recent research suggests that all 5 EC domains are required for optimal adhesion [65].

712 **Figure 2: Cadherin switching in bladder UCs** (taken from [22]). **a)** E-cadherin is strongly expressed at the cell
713 membrane throughout the normal urothelium. Reduced expression is observed in a proportion of NMIBCs, and the
714 majority of MIBCs demonstrate either reduced expression or a complete absence of E-cadherin; **b)** P-cadherin is
715 expressed in the basal 1-2 layers of normal urothelium, and this pattern is preserved in the majority of NMIBCs. The
716 majority of MIBCs demonstrate strong P-cadherin expression throughout the tumour mass; **c)** N-cadherin is not
717 expressed in normal urothelium or the majority of NMIBCs. However, the majority of muscle-invasive UCs express N-
718 cadherin throughout the tumour mass.

719 **Figure 3: Proposed pathways for the development of a bladder cancer stem cell phenotype and the relationship**
720 **with EpCAM** (adapted from [23]). Cancer stem cells (CSCs) result in the development of treatment resistant disease
721 in some cancer settings, and this diagram proposes potential pathways for their development in UBC. There is likely
722 considerable plasticity in these pathways [142], with cells reverting to a less aggressive state by mesenchymal-to-
723 epithelial transition (MET) or by the reversal of the CSC phenotype, and most likely influenced by the tumour
724 microenvironment [23]. We also propose a model whereby EpCAM modulates the development of EMT and/or CSCs
725 (see text).

726 **Figure 4: EpCAM's relationship with E-cadherin** (adapted from [123;149]). The dual role of EPCAM in epithelial
727 tissues is demonstrated. EpCAM can either disrupt the adherens junction, resulting in the release of β -catenin (**a**), or
728 stabilise the adherens junction to maintain E-cadherin's anchorage to the cell cytoskeleton (**b**). In (a), released β -
729 catenin subsequently forms a complex with EpICD and the transcriptional co-factor FHL2 [150], either at the cell
730 membrane or in the cell nucleus. The EpICD/FHL2/ β -catenin complex then interacts with the Lef-1 transcription

731 factor in the cell nucleus to activate the transcription of various target genes, including known oncogenes. In UBC we
732 demonstrated that the extracellular domain of EpCAM is released by cleavage immediately adjacent to the cell
733 membrane [52]. The exact location of cleavage was not described by Maetzel et al [123], but the protease involved
734 (TACE or ADAM 17) usually cleaves membrane proteins 10-15 residues away from the membrane surface [127],
735 suggesting atypical cleavage or an alternative mechanism of extracellular domain release in UBC. (α = α -catenin, β = β -
736 catenin).

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Figure 1: Cell-cell adhesion in epithelial tissues.

Figure a

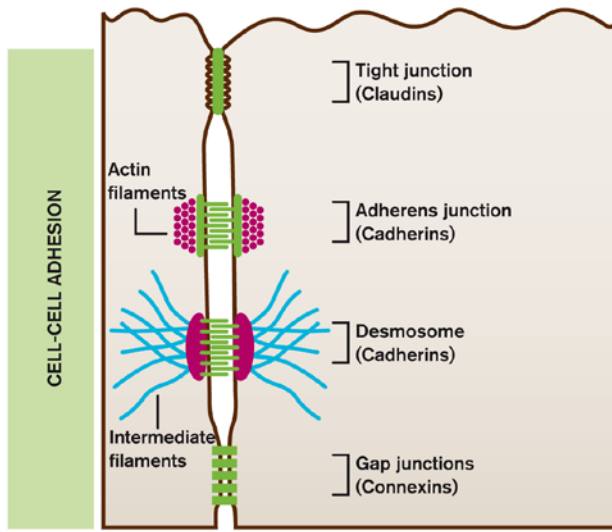


Figure b

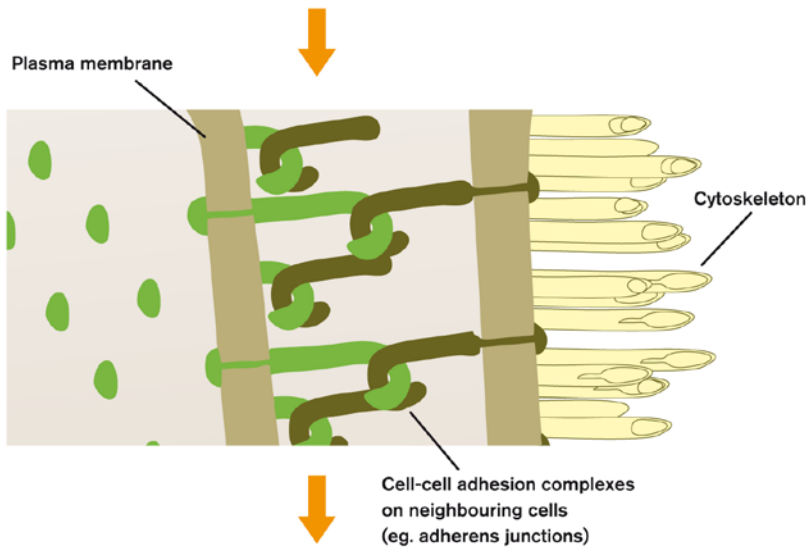


Figure c

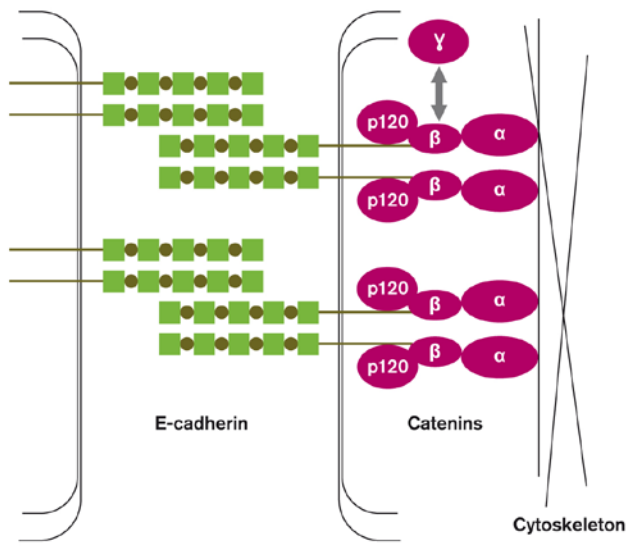
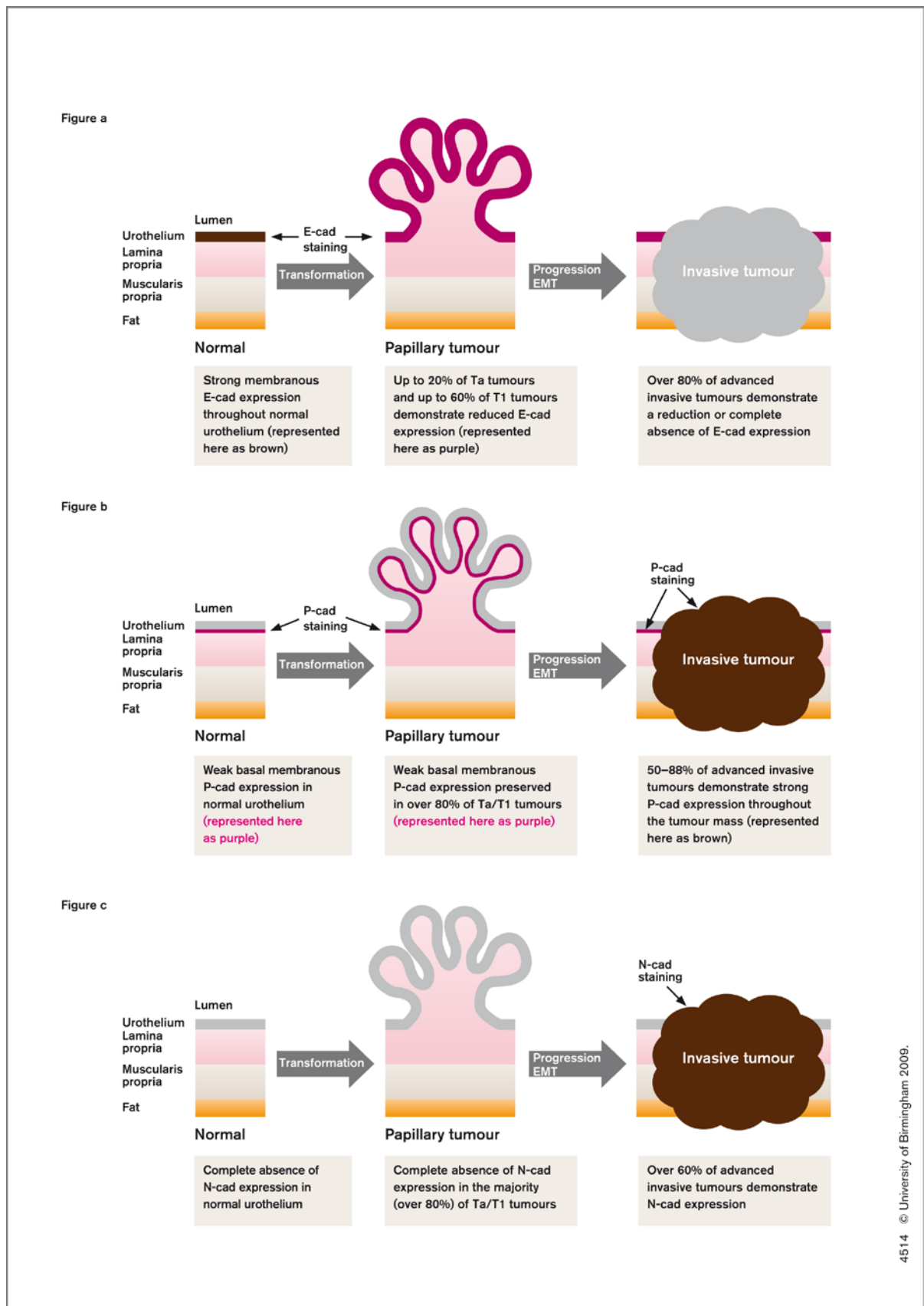
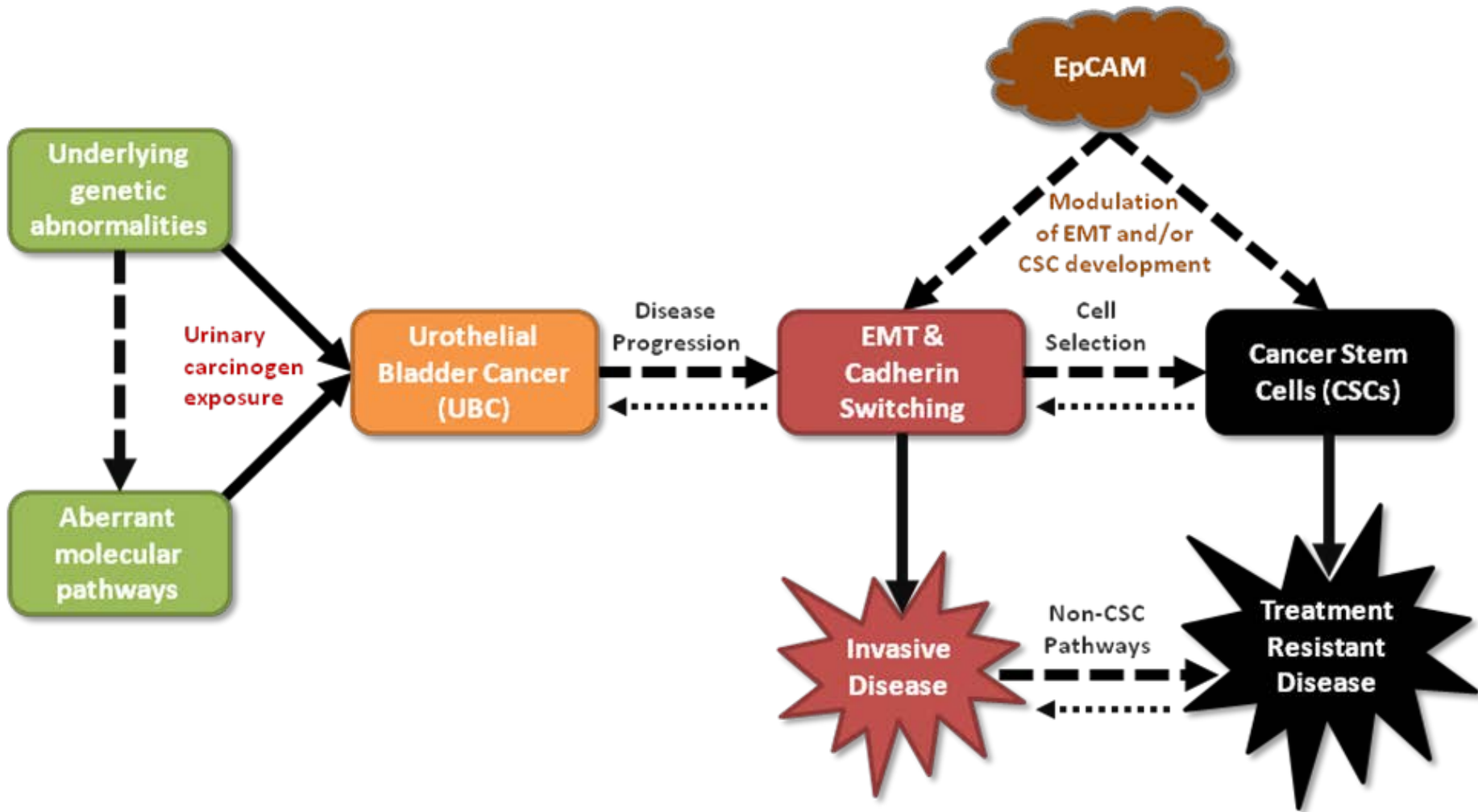


Figure 2: Cadherin switching in bladder UBCs.



744 Figure 3: Proposed pathways for the development of a bladder cancer stem cell phenotype and the relationship with EpCAM.

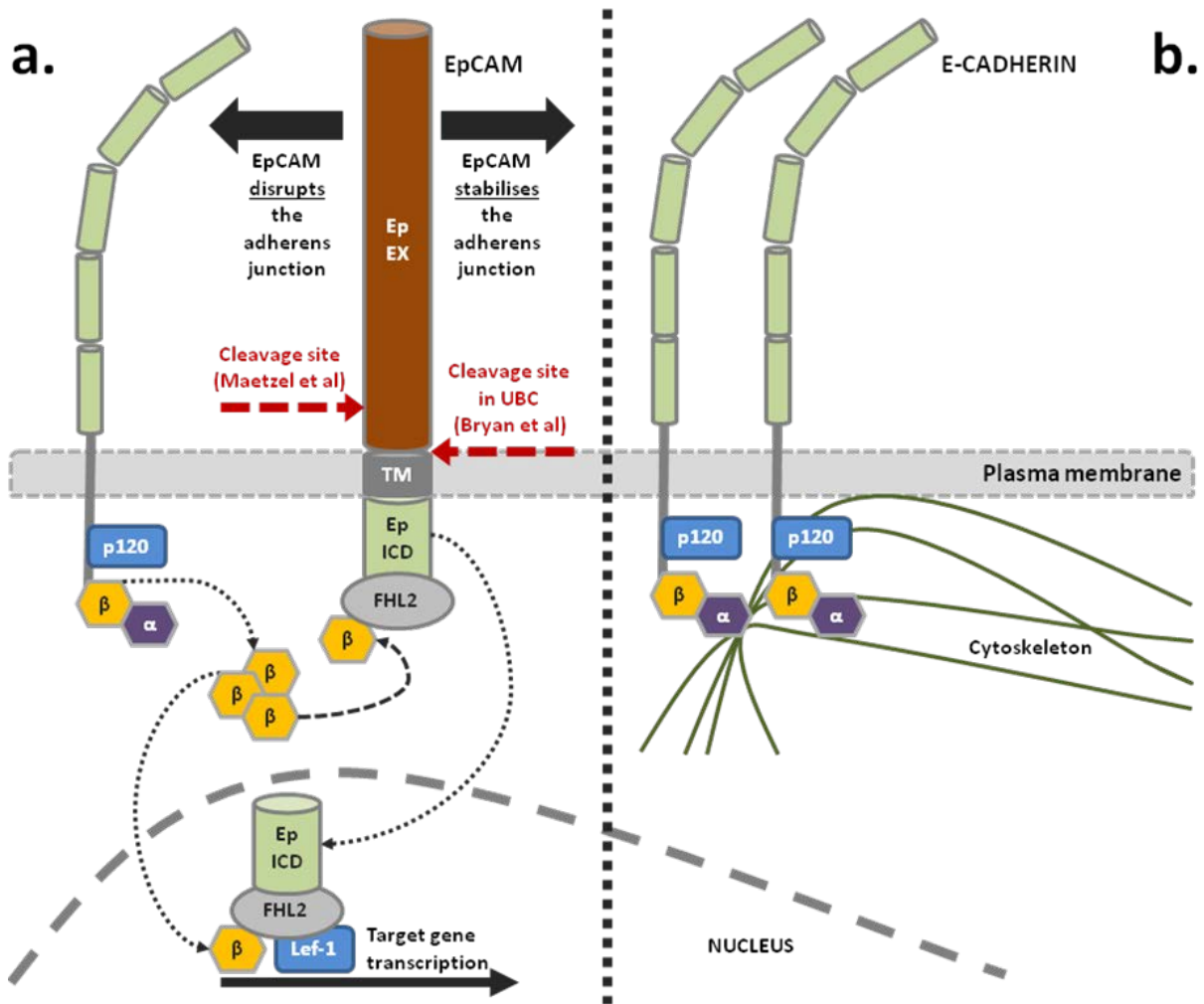
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747 Figure 4: EpCAM's relationship with E-cadherin.

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