Plasma membrane ion channels and epithelial to mesenchymal transition in cancer cells

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Running title: Ion channels and EMT in cancer

Abstract

A variety of studies have suggested that epithelial to mesenchymal transition (EMT) may be important in the progression of cancer in patients through metastasis and/or therapeutic resistance. A number of pathways have been investigated in EMT in cancer cells. Recently, changes in plasma membrane ion channel expression as a consequence of EMT have been reported. Other studies have identified specific ion channels able to regulate aspects of EMT induction. The utility of plasma membrane ion channels as targets for pharmacological modulation make them attractive for therapeutic approaches to target EMT. In this review, we provide an overview of some of the key plasma membrane ion channel types and highlight some of the studies that are beginning to define changes in plasma membrane ion channels as a consequence of EMT and also their possible roles in EMT induction.

Introduction

Epithelial to Mesenchymal transition (EMT) refers to the process whereby epithelial cells which typically exhibit features such as strong cell to cell adhesion and apical-basal polarity, lose these properties and acquire others such as greater motility and a spindle like morphology (van Denderen and Thompson 2013) (Thiery, et al. 2009) (Fig. 1). EMT is a key event in developmental processes including embryogenesis where it is associated with implantation and embryonic gastrulation (Kalluri and Weinberg 2009). EMT is also a feature of other aspects of normal physiology such as wound healing where it has an important role in tissue regeneration, and organ fibrosis (Kalluri and Weinberg 2009).

EMT in cancer

Metastasis is the cause of mortality in cancer types that originate from organs where surgical resection and/or treatment of the primary tumour are often feasible (e.g. breast and prostate). Metastasis is a highly regulated process whereby cells escape the primary tumour, enter the circulatory system and deposit at a metastatic site (Hanahan and Weinberg 2011). There is clear coordination of processes in metastasis and this is reflected in the propensity of different cancer subtypes to preferentially form metastatic lesions in specific sites. The loss of cell-to-cell adhesion, the acquisition of motility, the ability to degrade the surrounding extracellular matrix and to survive stresses such as that induced by entry into the circulation are all features that are required of cancer cells during metastasis. It is therefore not surprising that it is believed that as cells leave the primary tumour they may undergo processes similar to EMT (Heerboth, et al. 2015). These include the expression of the specific transcription factors Snail and Twist, expression of mesenchymal markers such as vimentin and N-Cadherin, and loss of epithelial markers such as E-cadherin (Tsai and Yang 2013). Indeed, the consequences of EMT have been reported as increased motility and a remodelling of cellular adhesion (Lamouille, et al. 2014). EMT in cancer cells is also associated with the acquisition of therapeutic resistance (Singh and Settleman 2010). Although some very recent studies

indicate that in some cancers EMT may be more important in the acquisition of therapeutic resistance than metastasis (Fischer, et al. 2015; Zheng, et al. 2015), understanding the induction of EMT and the properties of the mesenchymal state would clearly help identify novel therapeutic targets.

A number of factors in the tumour microenvironment have been identified as inducers of EMT in cancer cells. In breast cancer cells, growth factors such as epidermal growth factor (EGF), and hypoxia have been shown to induce EMT in a variety of *in vitro* models, such as MDA-MB-468 breast cancer cells and ZR-75-1 breast cancer cells (Davis, et al. 2014a; Lester, et al. 2007). In prostate cancer cells, EMT is induced by epidermal growth factor (EGF) (Zhang, et al. 2013b) and Growth and differentiation factor 9 (GDF-9) (Bokobza, et al. 2011). Studies in lung cancer cells have demonstrated that hypoxia induces EMT through protein kinase A (PKA) activity in a hypoxia-inducible factor 1-alpha (HIF1-α) dependent manner (Shaikh, et al. 2012). A variety of drugable targets have been identified as potential mechanisms to control EMT induction and/or target the mesenchymal phenotype which is a consequence of EMT (Davis, et al. 2014b). One class of proteins that are the target of existing drugs and many drug development programs are ion channels. Plasmalemmal ion channels in particular are often amenable to pharmacological modulation due to their extracellular domains. The availability of selective inhibitors to specific ion channel isoforms also allows chemogenomic and other methods to develop new therapeutics.

Ion channels as regulators of cellular processes

The presence of ion gradients across the plasma membrane is a defining feature of mammalian cells. The sodium ion gradient is maintained by Na^+/K^+-ATPases that actively pump Na^+ ions from the cytoplasm to maintain a lower intracellular free Na^+ level compared to those of the extracellular space (Castillo, et al. 2015). Changes in this gradient can lead to rapid changes in membrane potential and drive action potentials in excitable cells. Similarly, changes in cytosolic free Ca^{2+} ($[Ca^{2+}]_{CYT}$) levels can be mediated by activation of Ca^{2+} permeable ion channels and such changes

have important roles in an array of cellular processes including fertilization, muscle contraction, hormone secretion, gene transcription and cell death (Berridge, et al. 2003). The diversity of processes influenced by changes in Na^+, Ca^{2+} and K^+ and other ions through the opening of ion channels, requires the cell to selectively control such changes and the way such changes are decoded to alter cellular processes. Hence, it is not surprising that there are a plethora of ion channels in cells. For example there are over 20 genes that encode for just one specific class of ion channel - transient receptor potential (TRP) channels in humans. The next section provides an outline of the general properties of ion channels relevant to this review. We then provide a specific overview of studies that have identified roles of ion channels in EMT induction and/or remodelling.

Plasma membrane ion channels

There are a variety of ion channels with different permeability and selectivity for cations or anions. A comprehensive review of all ion channels even just those of the plasma membrane is well beyond the scope of this review. Hence, readers are directed to sources of comprehensive lists and review of ion channels such as the IUPHAR/BPS guide to pharmacology (Southan, et al. 2016), which includes other channels not discussed in this review such as acid-sensing (proton-gated) ion channels (ASICs) and some ligand gated Ca^{2+} channels such as ionotropic glutamate receptors. Arguably, the most extensively studied plasma membrane ion channels are those depicted in Fig. 2 – which include calcium channels, sodium channels, potassium channels and chloride channels. Examination of each of these channel types provides insight into their diversity. These channels can differ dramatically in their properties from ion selectivity to their mechanism of activation.

The diversity in ion channel properties is clear in the plasma membrane Ca^{2+} channels presented in Fig. 2 – Orai, TRP, P2X and voltage gated Ca^{2+} channels (VGCC). These classes have clear differences in their mechanism of activation. For example the Orai1 protein is part of a complex whereby Ca^{2+} influx is activated by the depletion of endoplasmic reticulum Ca^{2+} stores (Azimi, et al. 2014). In contrast, TRP channels have been described as sensors, as exemplified by TRPV1 a Ca^{2+} permeable

ion channel activated by heat and the hot chilli component, capsaicin (Azimi et al. 2014). Other ligand gated calcium channels include ionotropic glutamate receptors and also P2X channels that are activated by some nucleosides (e.g. ATP) whereas VGCCs are activated by changes in membrane potential (Azimi et al. 2014). Even within classes of Ca^{2+} channels there is great diversity of activators (e.g. TRPV1 is activated by capsaicin whereas TRPM8 is activated by menthol) and ion selectivity (e.g. TRPV6 is highly selective for Ca^{2+} ions whereas TRPV1 is also permeable to Na^{+} ions) (Azimi et al. 2014). The remodelling of Ca^{2+} channel expression has been defined in some cancers and some have been identified as potential therapeutic targets in some cancer subtypes as reviewed elsewhere (Azimi et al. 2014; Stewart, et al. 2015). Indeed, SOR-C13, a TRPV6 inhibitor has been recently assessed in clinical trials of ovarian cancer (www.clinicaltrials.gov, NCT01578564).

Although the association between Na^{+} influx and action potentials has seen a focus on Na^{+} channel in neuroscience and cardiovascular research, Na^{+} channels are in fact expressed in a variety of cell types. For example voltage gated sodium channels (VGSC) are expressed in excitable cells including neurons and muscle cells, where they are responsible for action potential and conduction (Southan et al. 2016); NALCN has been described as a sodium leak channel which regulates the resting membrane potential and excitability in neurons (Cochet-Bissuel, et al. 2014); and epithelial sodium channels (ENaC) play pivotal roles in the regulation of extracellular fluid (ECF) volume and blood pressure in kidney tubules (Hanukoglu and Hanukoglu 2016). Potassium channels are equally as complex and diverse and include those that are voltage gated (VGKC), those that are two-pore domain (K2P), those that play roles in Ca^{2+}-activated K^{+} transport (KCa channels) and Inwardly rectifying K^{+} (IRK) channels (Hibino, et al. 2010).

Chloride channels include channels that when defective due to hereditary mutation can alter the fluid transport in epithelial cells resulting in cystic fibrosis (Cystic Fibrosis Transmembrane conductance Regulator (CFTR)), channels activated by intracellular Ca^{2+} (CaCC), those which are

ligand activated (LGCC), or volume regulated (VRAC) or the chloride channel superfamily (CIC) (Southan et al. 2016).

The outline of plasmalemmal ion channels presented above highlighted the diversity of ion channels and their roles in mammalian cells. As discussed below, some of these ion channels have recently been shown to be remodelled as a consequence of EMT in cancer cells or play roles in the induction of EMT markers induced by some stimuli.

Plasma membrane ion channels and EMT in cancer cells

This review is focused on the remodelling and in some cases roles of ion channels in EMT in cancer cells. It should be noted that other studies have investigated ion channels in EMT in the context of other EMT relevant processes many of which intersect with disease states such as airway remodelling (Arthur et al., 2015) and renal fibrosis (Mai et al., 2016).

The very different properties of cancer cells such as the acquisition of therapeutic resistance and the major changes in the expression of specific proteins (e.g. vimentin) and transcription factors (e.g. twist and Snail) as a consequence of EMT means that changes in ion influx should not have been surprising. The change in phenotype of cancer cells that have undergone EMT and the very specific roles of specific ion channels in different cell types suggests that the mesenchymal phenotype will exploit different ion channels to achieve different cellular functions. In the section below we will provide an overview of studies that have now shown such changes and in some cases implicated specific ion channels in EMT induction. Many of these studies are summarised in Table 1.

Sodium channels and EMT in cancer cells

Hypotheses have been proposed and an intellectual case made for the potential of voltage-gated sodium channels to regulate EMT induction in cancer cells (Eren and Oyan 2014; Eren, et al. 2015). The repositioning of clinically used voltage-gated sodium channel blockers to attenuate metastatic

progression and/or chemotherapy resistance through inhibition of EMT induction has also been highlighted (Eren et al. 2015). However, this area has yet to be fully assessed experimentally with models of EMT in cancer cells, and this represents an opportunity for future research.

Potassium channels and EMT in cancer cells

The association between changes in the potassium gradient and EMT was suggested in early studies of potassium chloride co-transporter 3 (KCC3) (Hsu, et al. 2007). KCC3 is not an ion channel, but its ability to cotransport K^+ and Cl^- ions makes it an important regulator of the flux of these ions across the plasma membranes of many cell types where it can play an important role in the regulation of cell volume (Hsu et al. 2007; Kahle, et al. 2015). Forced overexpression of KCC3 in cervical cancer SiHa cells is associated with the adoption of a more mesenchymal-like morphology, the down regulation of the epithelial marker E-cadherin and the upregulation of the mesenchymal marker vimentin (Hsu et al. 2007). Subsequent to these studies an association with the EAG1 potassium channel and EMT in lung cancer cells has been implicated, because of an increase in Eag1 mRNA levels in A549 lung cancer cells treated with transforming growth factor beta 1 (TGFβ1), an EMT inducer in this model (Restrepo-Angulo, et al. 2011). In context of colorectal cancer, studies of phosphatase of regenerating liver-3 (PRL-3) induced EMT in LoVo cells (a colon cancer cell line), has shown that a pharmacological inhibitor of the Ca^{2+} activated potassium channel KCNN4 - TRAM-34, supresses the mesenchymal markers vimentin and Snail, and increases the expression of the epithelial marker E-cadherin (Lai, et al. 2013). Although the concentrations of TRAM-34 used may have inhibited other ion channels, siRNA to KCNN4 phenocopied the effects of TRAM-34 (Lai et al. 2013). Moreover, KCNN4 expression was positively correlated with tumour stage in clinical cohort of 86 patient colorectal tumour samples (Lai et al. 2013). Very recent studies have now shown that silencing of KCNN4 in MDA-MB-231 (a breast cancer cell line with features of the mesenchymal phenotype) appeared to reduce the expression of the mesenchymal markers vimentin and Snail1 (Zhang, et al. 2016).

Collectively, the studies described above are beginning to define associations between specific potassium channels and EMT in cancer cells. Further studies of other potassium channels in the context of changes in expression as a consequence of EMT as well as the induction of EMT and/or maintenance of the mesenchymal phenotype now seem appropriate. Given the diversity of EMT models in cancer cells and the variety of inducers of EMT, it is also important that the roles of specific potassium channels be investigated across a variety of models and inducers of EMT.

Chloride channels and EMT in cancer cells

An increasing number of studies have identified the remodelling of expression of chloride channel components as a consequence of EMT in cancer cells. Examples of such remodelling include isoforms of chloride channel accessory proteins, namely CLCA2 and CLCA4. CLCA2 mRNA levels are reduced in breast cancer cell lines associated with the mesenchymal phenotype (e.g. MDA-MB-231 and BT549) compared to those often enriched in epithelial markers (e.g. MCF-7). Indeed, expression of the EMT transcription factor Snail supresses CLCA2 protein in the human breast cell line MCF10A, and CLCA2 levels are reduced in subpopulations of cells from the human mammary epithelial (HMLE) cell line that are enriched in mesenchymal markers (Walia, et al. 2012). Moreover, CLCA2 levels are reduced during EMT induced by TGFβ (Yu, et al. 2013). Similarly, reduced levels of the related isoform CLCA4 is a feature of subpopulations of cells from the HMLE cell line that are enriched in mesenchymal markers and a consequence of TGFβ-induced EMT (Yu et al. 2013). Consistent with the loss of CLCA2 and CLCA4 in the mesenchymal phenotype, low levels of CLCA2 and CLCA4 appear likely to be associated with an increased incidence of metastasis (as assessed through metastasis or relapse free survival) using specific cohorts of breast cancer patients (Walia et al. 2012; Yu et al. 2013). In addition to their remodelling as a consequence of EMT, CLCA2 and CLCA4 have also been implicated in the regulation of the transition of breast cancer cells towards a more mesenchymal state. Knockdown of CLCA2 or CLCA4 is sufficient in HMLE cells to induce the expression of the mesenchymal marker vimentin and supress the epithelial marker E-Cadherin (Walia et al. 2012; Yu et

196 al. 2013). In the case of CLCA2, the regulation of EMT may at least in part be through interactions
197 with the cell junctional protein EVA1 (Ramena, et al. 2016). Future studies are now required to
198 define the relative importance in changes in chloride flux in these events, and the ability of the loss
199 of CLCA2 or CLCA4 to induce a mesenchymal phenotype in other models of EMT, including those not
200 of breast cancer origin.

201 Breast cancer cells have also been the focus of investigators exploring the relationship between
202 CFTR and EMT. The EMT inducer TGFβ1 causes a down regulation of CFTR in MCF-7 cells, which is
203 also associated with a down regulation of the epithelial marker E-cadherin (Zhang, et al. 2013a). A
204 functional role for CFTR in EMT induction is suggested by the ability of CFTR silencing to induce the
205 expression of a variety of mesenchymal markers in MCF-7 breast cancer cells. This proposed function
206 of CFTR is further supported by the ability of CFTR overexpression in mesenchymal-like MDA-MB-
207 231 breast cancer cells to suppress the expression of vimentin (a mesenchymal marker) and induce
208 the expression of E-cadherin (an epithelial marker) (Zhang et al. 2013a). As would be predicted
209 based on these results, reduced levels of CFTR are associated with poor prognosis in breast cancer
210 patients (Zhang et al. 2013a). More recent studies have begun to explore chloride channels in the
211 context of EMT in other cancer types, such as squamous cell carcinomas of the head and neck
212 (Shiwarski, et al. 2014). TMEM16A (also known as ANO1), is one of a reported subset (termed
213 Anoctamins) of calcium activated chloride channels (Kunzelmann, et al. 2011). Levels of TMEM16A
214 are reduced in cancer cells in metastatic lymph nodes compared to those of the primary tumour in
215 squamous cell carcinomas of the head and neck (Shiwarski et al. 2014). TMEM16A seems to be
216 more than a potential marker of EMT, since silencing of TMEM16A in T24 cells (a human bladder
217 carcinoma cell line), produces a mesenchymal-like phenotype (spindle morphology, lower E-
218 cadherin, increased Snail) and overexpression of TMEM16A produces an epithelial-like phenotype
219 (rounded packed morphology, increased E-cadherin, reduced vimentin and fibronectin) (Shiwarski
220 et al. 2014).

The work described above, performed by a variety of investigators using an array of models and approaches has now helped define a remodelling of specific chloride channels (or components) in EMT and a role for these same channels in the induction of EMT and/or the maintenance of the epithelial-like phenotype.

Calcium channels and EMT in cancer cells

The calcium signal has been identified as or could be speculated to be a potential mechanism by which at least some of the aforementioned ion channels may immediate their effects on EMT. For example the mechanism by which KCNN4 may regulate EMT in colon cancer cells has been linked to effects on calcium signalling (Lai et al. 2013). Indeed, global chelation of intracellular free Ca^{2+} that attenuates increases in cytosolic free Ca^{2+}, suppresses both EGF and hypoxia induced increases in the mesenchymal markers vimentin, N-cadherin and CD44 (Davis et al. 2014a) . Similar findings have now been reported in Huh7 and HepG2 hepatic cancer cell lines for EMT induced by doxorubicin (Wen, et al. 2016). It is also now clear that a major remodelling in calcium signalling and the expression of specific calcium permeable ion channels is a feature of EMT and some calcium permeable ion channels are important in the induction of expression of some proteins associated with the mesenchymal phenotype.

Alterations in the responses to ATP, able to activate G-protein coupled purinergic receptors (P2Y family) and ligand gated Ca^{2+} channels (P2X family) is a feature of both EGF and hypoxia induced EMT in MDA-MB-468 breast cancer cells (Azimi, et al. 2015; Davis, et al. 2011). EMT induced by hypoxia and EGF is associated with the attenuation of peak $[Ca^{2+}]_{CYT}$ and the sustained phase of Ca^{2+} influx induced by ATP. EMT is also associated with a reduction in the sensitivity to ATP with an increase the EC_{50} (Azimi et al. 2015; Davis et al. 2011). Such changes in the mesenchymal phenotype may be an adaption of breast cancer cells to the high ATP concentrations in some tumour microenvironments. However, despite this consistent change in ATP-mediated Ca^{2+} signalling, the nature of the remodelling of P2X receptors seems very different as the upregulation of P2X5 mRNA is a feature of

246 EGF but not hypoxia associated ATP (Azimi et al. 2015; Davis et al. 2011). The attenuation of store
247 operated Ca^{2+} entry (SOCE) and basal Ca^{2+} influx is also a feature of EGF induced EMT in MDA-MB-
248 468 (Davis, et al. 2012), however, assessment of such changes with hypoxia induced EMT has not
249 been reported. Such studies are critical given that in MCF-7 cells, the EMT inducer TGF-β1 has been
250 reported to be associated with enhancement of store operated Ca^{2+} entry (Hu, et al. 2011).

251 In addition to a remodelling of Ca^{2+} influx and/or the expression of some Ca^{2+} permeable ion
252 channels in EMT in cancer cells, specific calcium permeable ion channels have also been identified as
253 regulators of the induction of at least some hallmarks of EMT. A focused siRNA screen identified
254 TRPM7 as a regulator of EGF-induced expression of the mesenchymal marker vimentin in MDA-MB-
255 468 breast cancer cells (Davis et al. 2014a). A pharmacological inhibitor of TRPM7 replicated the
256 consequences of TRPM7 silencing on EGF induced vimentin expression. These effects were not due
257 to general inhibition of EGF receptor (EGFR) signalling since EGF-mediated EGFR and AKT
258 phosphorylation were unaffected by TRPM7 silencing, however, EGF-mediated STAT3 and ERK1/2
259 phosphorylation were significantly reduced (Davis et al. 2014a). Although a Ca^{2+} permeable ion
260 channel, the importance of TRPM7 in Mg^{2+} homeostasis and its ability to function as an atypical
261 alpha kinase (Paravicini, et al. 2012) require further attention into the nature of its contribution to
262 EMT in some cancer models. Silencing of the cold sensor TRPM8 increases the expression of the
263 epithelial marker E-cadherin in mesenchymal-like MDA-MB-231 cells and reduces levels of the
264 mesenchymal marker vimentin (Liu, et al. 2014). Consistent with a role for TRPM8 in the
265 maintenance and/or induction of the mesenchymal phenotype in breast cancer cells, overexpression
266 of TRPM8 in the more epithelial like MCF-7 cell line leads to EMT induction as indicated by down
267 regulation of E-cadherin and induction of vimentin (Liu et al. 2014). In Huh7 and HepG2 hepatic
268 cancer cells, TRPC6 silencing attenuates changes in the expression of E-cadherin induced by
269 doxorubicin suggesting that in the ability of TRPC6 silencing to increase sensitivity to doxorubicin
270 through effects of resistance pathways may be due at least in part to effects on some aspects of EMT
271 induction (Wen et al. 2016).

Hence, studies of calcium signalling and Ca^{2+} permeable ion channels in EMT from a variety of groups using an array of EMT inducers and models have helped define a critical role for the Ca^{2+} signal in EMT in cancer cells.

Conclusion

An increasing number of studies have reported the remodelling of plasma membrane ion channel expression as a characterizing feature of EMT in cancer cells. The identification of the role of specific ion channels in the induction of EMT and/or the maintenance of aspects of the epithelial or mesenchymal-like phenotype in cancer cells suggest that some ion channels may be therapeutic targets to control EMT and hence disease progression (e.g. therapeutic resistance). However, it is likely that different EMT inducers may engage different ion channels to regulate the properties of the mesenchymal phenotype and/or EMT induction itself. This issue and the study of the intersection between sex hormones and receptors that regulate EMT (Jeon, et al. 2016; Kong, et al. 2015; Zuo, et al. 2010; Sun, et al. 2014; van der Horst, et al. 2012) and ion channels which themselves intersect with sex hormone pathways (Asuthkar, et al. 2015; Hao, et al. 2015; Mahmoodzadeh, et al. 2016) are areas for future research. Which ion channels to pursue for therapeutic targeting requires careful consideration, and deciding factors will include the expression of targets in other cell types and the likely adverse systemic effects of channel inhibitors. However, the successful use of ion channel inhibitors for conditions as diverse as cardiovascular disease to pain demonstrated the need to continue research in this area.

Declaration of Interest

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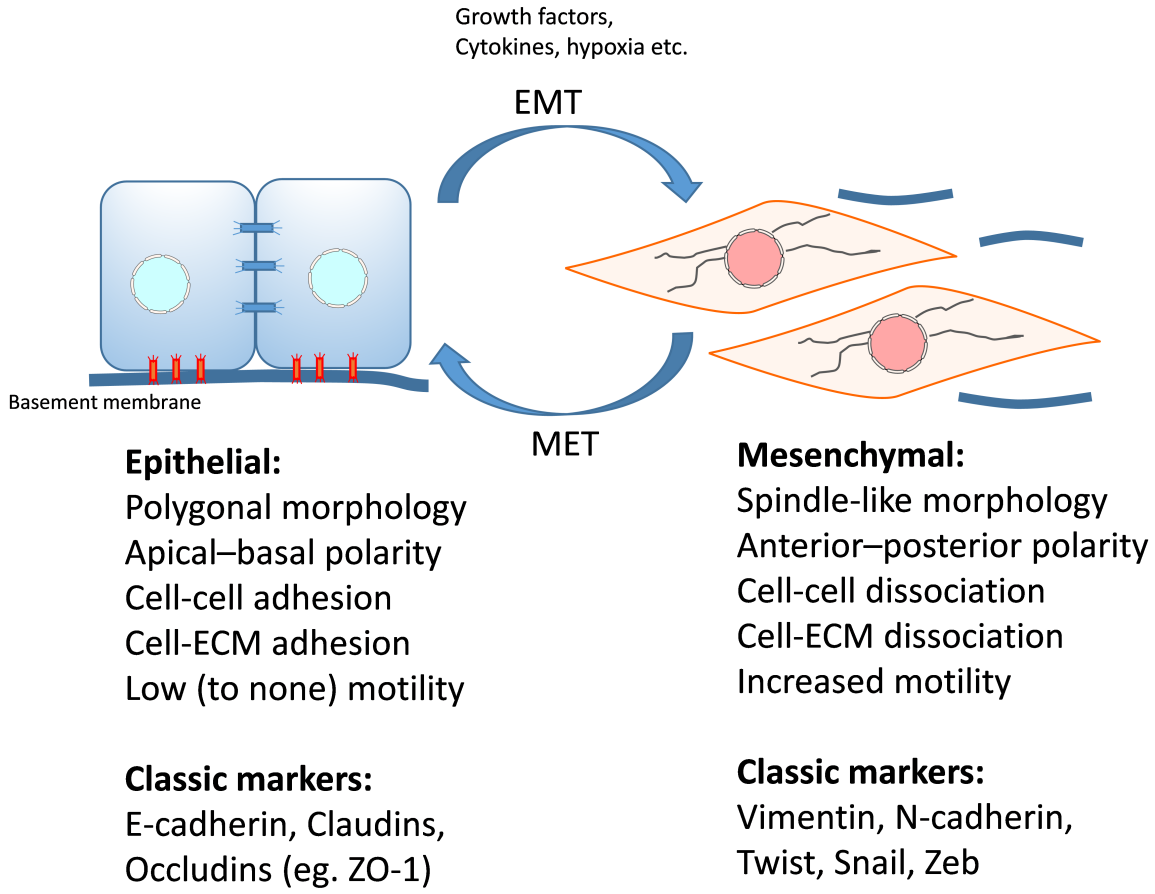

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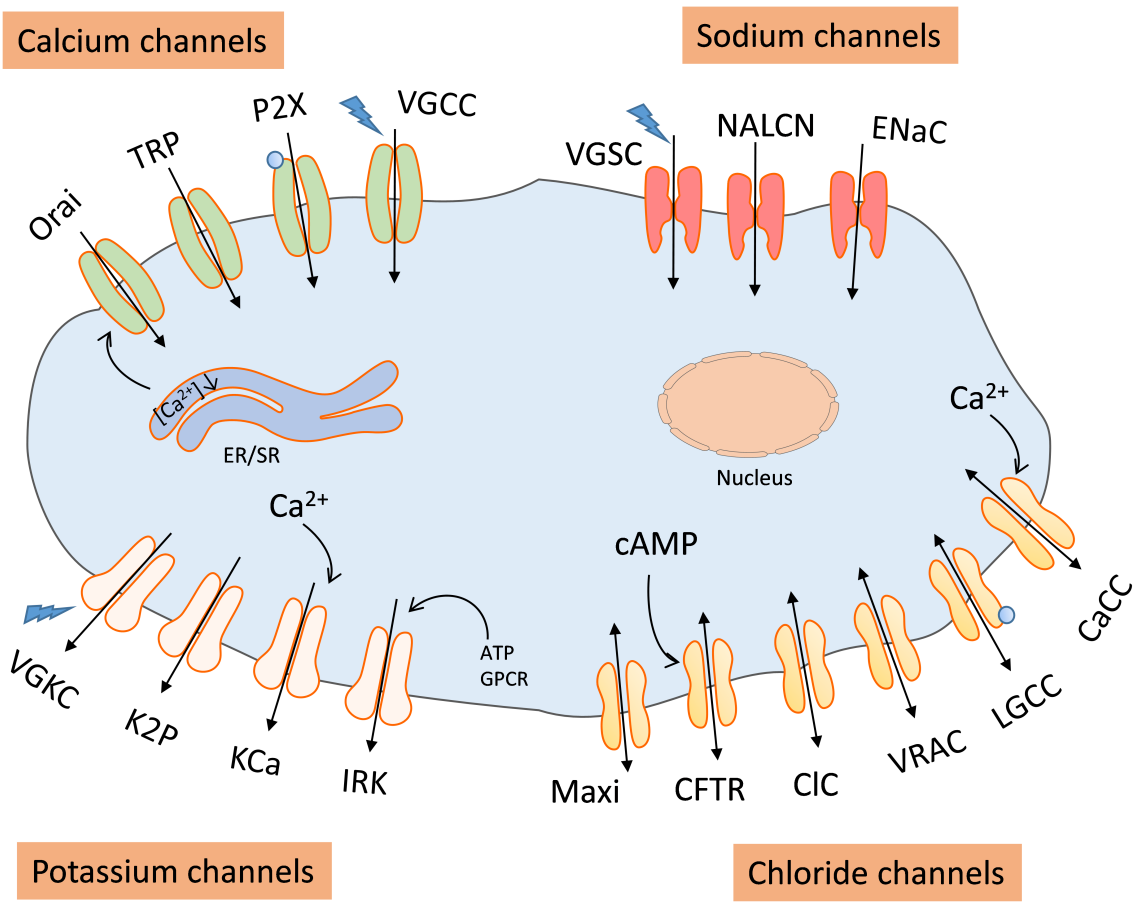
Calcium channels
Sodium channels
P2X
VGCC
TRP
Orai
VGSC
NALCN
ENaC
[Ca2+]v
ER/SR
Nucleus
Ca2+
Ca2+
cAMP
ATP
GPCR
VGKC
K2P
KCa
IRK
Maxi
CFTR
ClC
VRAC
LGCC
CaCC
Potassium channels
Chloride channels

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