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Quantitative methods for analyzing cell–cell adhesion in development

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ABSTRACT

During development cell–cell adhesion is not only crucial to maintain tissue morphogenesis and homeostasis, it also activates signalling pathways important for the regulation of different cellular processes including cell survival, gene expression, collective cell migration and differentiation. Importantly, gene mutations of adhesion receptors can cause developmental disorders and different diseases. Quantitative methods to measure cell adhesion are therefore necessary to understand how cells regulate cell–cell adhesion during development and how aberrations in cell–cell adhesion contribute to disease. Different *in vitro* adhesion assays have been developed in the past, but not all of them are suitable to study developmentally-related cell–cell adhesion processes, which usually requires working with low numbers of primary cells. In this review, we provide an overview of different *in vitro* techniques to study cell–cell adhesion during development, including a semi-quantitative cell flipping assay, and quantitative single-cell methods based on atomic force microscopy (AFM)-based single-cell force spectroscopy (SCFS) or dual micropipette aspiration (DPA). Furthermore, we review applications of Förster resonance energy transfer (FRET)-based molecular tension sensors to visualize intracellular mechanical forces acting on cell adhesion sites. Finally, we describe a recently introduced method to quantitate cell-generated forces directly in living tissues based on the deformation of oil microdroplets functionalized with adhesion receptor ligands. Together, these techniques provide a comprehensive toolbox to characterize different cell–cell adhesion phenomena during development.

Introduction

Cell–cell adhesion in development

During embryonic development the ability of cells to adhere to one another is fundamental for the assembly of a three-dimensional tissue and forms the basis for the formation of multicellular organisms. Cell–cell adhesion is not only important to simply keep cells together but also to organize them in complex tissues with diverse and distinctive patterns. A first significant demonstration of the importance of cell–cell adhesion for germ layer assembly was made by Townes and Holtfreter in the 1950s. Using dis- and re-association assays of amphibian embryonic cells and tissues, the authors demonstrated that randomly intermixed cells spontaneously self-organize to reconstitute the different germ layers. Interestingly, the rearranged tissues reflected the same arrangement as native tissues during normal embryonic development, whereby the ectoderm is located in the periphery, the endoderm is internal, and the mesoderm is arranged in the region between them (Barriga et al., 2013). Holtfreter termed this phenomenon as 'selective affinity', although the mechanism providing the driving force underlying these cell and tissue rearrangements was not clear defined. Later, Steinberg attributed this phenomenon to differential cell–cell adhesion processes and proposed the 'differential adhesion hypothesis' (DAH) (Foty and Steinberg, 2013; Steinberg, 1996, 2007). DAH defines that tissues behave like unmixable liquids with a given surface tension and that differences in tissue surface tension control cell segregation and tissue organization. Furthermore, Steinberg proposed that tissue surface tension scales with cell adhesion so that differences in cell adhesion among different cell types drives tissue segregation. DAH was verified experimentally both in the developing *Drosophila* retina and in cell culture (Foty and Steinberg, 2005; Hayashi and Carthew, 2004). Moreover, several studies demonstrate the importance of differential adhesion during morphogenesis, including *e.g.* cell sorting in the *Drosophila* imaginal wing disc (Chang et al., 2011; Dahmann and Basler, 2000), rhombomere domain boundary formation (Cooke et al., 2005), gastrulation movements (Maitre et al., 2012; Ninomiya et al., 2012; Shimizu et al., 2005) and neural crest migration (Mayor and Theveneau, 2013; McKeown et al., 2013). However, DAH has remained

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controversial and alternative hypotheses have been suggested to explain cell sorting. For instance, Harris proposed the differential surface contraction model (DSC) in which cell sorting is driven by differences in actomyosin-dependent cortical tension, rather than by cell–cell adhesion *per se* (Harris, 1976). Subsequently, the differential interfacial tension hypothesis (DITH) was introduced by Brodland which combines elements from both DAH and DSC theories (Brodland, 2002). The DITH postulates that cell rearrangements are controlled by interfacial tension, which largely depends on both cell adhesion and cell contraction. Taken together, it should be emphasised that a balance between cell–cell adhesion, cortical tension and cortical elasticity promote surface and interfacial tension as discussed in different studies (Farhadifar et al., 2007; Foty and Steinberg, 2013; Krieg et al., 2008a; Lecuit and Lenne, 2007; Paluch and Heisenberg, 2009), although the exact interplay between these mechanisms remains elusive.

Cadherins

A key element in the regulation of tissue morphogenesis is the formation, rearrangement and maintenance of physical cell–cell contacts mediated by different adhesion molecules and cell surface ligand and receptor systems (Yamada and Nelson, 2007). Several classes of adhesion molecules, including members of the immunoglobulin superfamily (Cunningham, 1995), selectins (Rosen and Bertozzi, 1994) and cadherins mediate cell–cell adhesion and control the physical interactions between cells. This review focuses primarily on methods to study adhesion mediated by cadherin receptors, one of the most comprehensively studied families of cell–cell adhesion receptors in a developmental context. Cadherins form a multigene family of Ca^{2+}-dependent glycoproteins promoting homotypic cell–cell adhesion in most animal species (Oda and Takeichi, 2011). Cadherins are particularly important for the dynamic regulation of adhesive contacts and they are therefore crucial for promoting diverse morphogenetic processes. Intense research into cadherin function started in the early 1980s, when Jacob and co-workers described the role of E-cadherin (uvomorulin) in blastomeres compaction of an early developing mouse embryo (Hyafil et al., 1981; Peyrieras et al., 1983).

Classical cadherins are transmembrane proteins that mediate cell–cell adhesion by forming intracellular bonds through interactions of their extracellular sub-domains on opposed cells (trans-orientation) by a mechanism called strand swapping (Posy et al., 2008; Zhang et al., 2009). The cytoplasmic domain of classical cadherins contains a β-catenin binding site, which dynamically links cadherins to the actin cytoskeleton via α-catenin (Drees et al., 2005; Pokutta et al., 2008). Anchoring of cadherins to the cytoskeleton is also promoted by recruitment of actin-binding proteins, such as epithelial protein lost in neoplasm (EPLIN) and vinculin (Abe and Takeichi, 2008; Alfandari et al., 2010). Importantly, the recruitment of actin-binding proteins induces remodelling of the underlying cortical cytoskeleton, with consequential changes in the mechanical properties of the cells (le Duc et al., 2010; Liu et al., 2010; Taguchi et al., 2011; Yonemura et al., 2010). In recent years several studies have focused on biophysical descriptions of cadherin function in cell–cell contact formation during morphogenesis, including aspects of interfacial tension, signalling to the actomyosin cytoskeleton and the mechanical coupling of contacting cells (Maitre and Heisenberg, 2013).

Apart from forming robust cell–cell contacts, cadherins mediate a number of intracellular signalling cascades that control cell proliferation (Kim et al., 2011; Nelson and Chen, 2003), cell polarity (Bosveld et al., 2012; Wang et al., 2010) and cell fate specification (Lorthongpanich et al., 2012; Stephenson et al., 2010). Moreover, cadherins modulate cell sorting, cell cortex tension, and promote cell migration of different cell types (Becker et al., 2012; Foty and Steinberg, 2004; Halbleib and Nelson, 2006; Maitre et al., 2012; Niessen et al., 2011; Takeichi, 1995). Dysregulation of cadherin adhesion and signaling function on the other hand leads to a broad variety of pathological defects including tumor invasion and metastasis (Berx and van Roy, 2009), inflammatory diseases (Hermiston and Gordon, 1995; Karayiannakis et al., 1998; Lee et al., 2007) or causes congenital defects in organogenesis (El-Amraoui and Petit, 2010).

During development different cell types migrate through the embryo and this requires constant modulation of cell–cell adhesion. One cell population that displays high motility during development is the neural crest (NC), a multipotent and highly motile cell population specific for vertebrates (Mayor and Theveneau, 2013). Importantly, NC cells (NCC) migrate collectively as a cohesive tissue. This recognized mode of migration also occurs in border cell migration in *Drosophila* and lateral line migration in zebrafish, as well as in wound healing and cancer metastasis (Friedl and Gilmour, 2009; Rorth, 2009). However, after a distinct time NCC progressively dissociate from the cell sheet and migrate as single cells until they find their final destination (Alfandari et al., 2010). Thus, cell–cell adhesion has to be precisely and continuously modulated during NCC migration. Interestingly, NCC not only display a similar migration behavior as invasive cancer cells, they also up-regulate a similar set of adhesion molecules, including cadherin-11 and N-cadherin (Tomita et al., 2000). NCC are therefore an excellent model system for investigating cell adhesion mechanisms underlying collective and single cell migration.

Another highly motile embryonic cell population are primordial germ cells (PGCs), which migrate as individual cells from the place where they are specified to the site of gonad formation (Richardson and Lehmann, 2010). Work in zebrafish provides evidence that E-cadherin mediated cell–cell adhesion is crucial for PGCs motility *in vivo* (Kardash et al., 2010). During migration PGCs form dynamic E-cadherin mediated contacts with neighboring somatic cells. PGCs then employ retrograde flow of actin-rich structures to exert pulling forces on these cadherin contacts, ultimately generating sufficient traction forces for the proper migration of these cells through the surrounding tissue (Kardash et al., 2010). Interestingly, a recent *in vitro* study using *Xenopus* PGCs demonstrates that pre-migratory PGCs exhibited stronger adhesion to somatic cells than migratory PGCs (Dzementsei et al., 2013). This observation is in direct correlation with the down-regulation of E-cadherin expression during PGC migration, which might contribute to the weakening of cell–cell adhesion contacts (Dzementsei et al., 2013). Thus, similar to NCC, cell–cell adhesion has to be precisely modulated for proper PGC migration.

In recent years advances in microscopy techniques and biophysical measurements have provided the possibility to identify biomechanical mechanisms underlying the formation and function of cell–cell contact, cell migration and tissue remodeling. Among these, different *in vitro* adhesion assays have been developed to characterize the adhesion strength between cells, which is generally measured by the ability of cells to remain attached to each when exposed to external forces. For instance, centrifugal assays in combination with radioactive cell labeling (Lotz et al., 1989; McClay et al., 1981) have been used to determine the formation kinetics of E-cadherin adhesion contacts in mouse fibroblasts (Angres et al., 1996), while shear flow assays are useful for studying adhesive interactions between endothelial cells and leukocytes (Kucik, 2009). Bulk adhesion assays offer the possibility to test a large number of cells, generating statistically relevant data within a short time frame. However, they determine the average behavior of cell populations and provide little information regarding the behavior of an individual cell (Chu et al., 2004). As a consequence, small differences in cell adhesion that are of potential biological significance are difficult to detect. For instance, adhesive

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