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ARTICLE

Rho-associated protein kinase regulates subcellular localisation of Angiomotin and Hippo-signalling during preimplantation mouse embryo development

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Abstract The differential activity of the Hippo-signalling pathway between the outer- and inner-cell populations of the developing preimplantation mouse embryo directs appropriate formation of trophectoderm and inner cell mass (ICM) lineages. Such distinct signalling activity is under control of intracellular polarization, whereby Hippo-signalling is either supressed in polarized outer cells or activated in apolar inner cells. The central role of apical-basolateral polarization to such differential Hippo-signalling regulation prompted us to reinvestigate the role of potential upstream molecular regulators affecting apical-basolateral polarity. This study reports that the chemical inhibition of Rho-associated kinase (Rock) is associated with failure to form morphologically distinct blastocysts, indicative of compromised trophectoderm differentiation, and defects in the localization of both apical and basolateral polarity factors associated with malformation of tight junctions. Moreover, Rock-inhibition mediates mislocalization of the Hippo-signalling activator Angiomotin (Amot), to the basolateral regions of outer cells and is concomitant with aberrant activation of the pathway. The Rock-inhibition phenotype is mediated by Amot, as RNAi-based *Amot* knockdown totally rescues the normal suppression of Hippo-signalling in outer cells. In conclusion, Rock, via regulating appropriate apical-basolateral polarization in outer cells, regulates the appropriate activity of the Hippo-signalling pathway, by ensuring correct subcellular localization of Amot protein in outer cells.

KEYWORDS: Angiomotin, Hippo-signalling, polarization, preimplantation stage embryo, Rho-associated protein kinase (Rock)

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Introduction

During preimplantation mouse embryo development, three distinct cell lineages are formed by the blastocyst stage. Two lineages constitute epithelialized and differentiating tissues that will give rise to post-implantation stage supportive extra-embryonic structures; namely, the outer-residing trophectoderm and the primitive endoderm (PrE) found at the boundary between the inner cell mass (ICM) and blastocoel. The third lineage, found deep within the ICM, is a pluripotent source for all fetal cells called the epiblast (EPI). As a prelude to the spatial separation of progenitor cells for the three-cell lineages, late 8-cell stage blastomeres enhance their adhesive properties to form adherens and tight junctions, leading to a compacted embryo, and initiate internal polarization by localising various polarity factors to either the outer-facing apical pole or basolateral regions in cell–cell contact (reviewed in, (Johnson, 2009; Rossant and Tam, 2009; Schrode et al., 2013; Zernicka-Goetz et al., 2009)). Examples of such factors are represented by apically localised partitioning-defective 3 (Pard3), partitioning-defective 6b (Pard6b) and atypical protein kinase C-zeta (Prkcz) and basolaterally distributed Scribble (Scrib) and partitioning-defective 1 (Mark2) (Alarcon, 2010; Plusa et al., 2005; Tao et al., 2012; Vinot et al., 2005). After subsequent cell cleavage division, resulting 16-cell stage daughter blastomeres can remain polarized on the outside surface of the embryo or they can become encapsulated inside, where they become apolar. The process of allocating daughter cells to differing spatial compartments is repeated in the following cleavage to yield 32-cell stage embryos exhibiting an outer population of trophectoderm progenitor cells and an ICM of PrE and EPI progenitors (Johnson and Ziomek, 1983), that then resolve into the three distinct and spatially organised lineages of the peri-implantation blastocyst (as reviewed/ discussed elsewhere, (Bruce, 2013; Johnson, 2009; Mihajlovic et al., 2015; Rossant and Tam, 2009; Schrode et al., 2013; Zernicka-Goetz et al., 2009)). Importantly, spatial cell allocation occurring during the two consecutive cleavages, can arise as the direct consequence of the division plane orientation, thus yielding either two outer cells or one outer and one inner cell, via 'conservative' or 'differentiative' divisions respectively (Johnson and Ziomek, 1981; Sutherland et al., 1990). Alternatively, atypical apolar outer cells can be physically forced inside the embryo by more polarized neighbouring outer cells (Anani et al., 2014).

The importance of an individual blastomere's relative position, either inside or on the outside of the developing embryo, in terms of the ultimate fate of its progeny, has been appreciated for some time (Hillman et al., 1972; Rossant and Lis, 1979; Spindle, 1978; Suwinska et al., 2008; Tarkowski and Wroblewska, 1967). The discovery of apical-basolateral polarity (Fleming and Johnson, 1988; Johnson and Ziomek, 1981, 1983), initiated a greater molecular understanding of how spatially distinct cells begin to differ, not least in their repertoire of expressed transcriptional regulators (see reviews, (Johnson, 2009; Rossant and Tam, 2009; Schrode et al., 2013; Zernicka-Goetz et al., 2009)). However, it has only been within recent years, with the discovery of the involvement of functional Hippo-signalling in conferring correct identity to emerging and spatially distinct trophectoderm and ICM lineages, that substantial molecular insight into the importance of cell position has come to the fore (reviewed in, (Sasaki, 2015)). In synopsis, the activity of Tead4, an essential trophectoderm-promoting transcription factor, is appropriately confined to outer cells and therefore blocked in inner cells, by the differential nuclear localisation of its required cofactors Yap1 and Taz (referred to here as Yap1, (Nishioka *et al.*, 2009)). This is achieved via a mechanism of actuated Hippo-signalling in the inner cell (at adherens junctions) that results in the activation of Lats1/2 kinases and in turn phosphorylates Yap1 to prevent its nuclear import and functional association with Tead4. In outer cells, the presence of functionally active apically localised polarity factors, inhibits Hippo-pathway activation via the direct sequestration of the Hippo-activator Angiomotin (Amot) to the apical pole. Consequently, Amot cannot associate with necessary downstream effectors, localised at adherens junctions, to initiate Lats1/2 dependent Yap1 phosphorylation, therefore permitting Yap1 to bind Tead4 in the nucleus (Cockburn et al., 2013; Hirate and Sasaki, 2014; Hirate et al., 2013; Leung and Zernicka-Goetz, 2013). Hence, in preimplantation embryos, the Hippo-signalling pathway integrates both cell position and polarity information in order to direct appropriate cell-lineage gene expression.

The Rho family of GTPases (Rho-GTPases), and their downstream effectors (*e.g.* Rho-associated kinases, Rock1/2), are associated with various cellular functions, including cell migration and adhesion, vesicular trafficking, establishment of apical-basolateral polarity and cytoskeletal dynamics (reviewed in, (Bustelo et al., 2007; Mack and Georgiou, 2014)). In the context of the preimplantation mouse embryo, a role for Rho-GTPases in regulating compaction and apical-basolateral polarization, has been known for some time (Clayton et al., 1999). More recent evidence from chemical inhibition of Rock, albeit at varying concentrations with differing phenotypic effects, detail defects in blastocoel formation, actin microfilament network, adoption of trophectoderm cell fate and cellular-apical-basolateral polarization and Hippo-signalling (Duan et al., 2014; Kawagishi et al., 2004; Kono et al., 2014).

Therefore, in the present study the aim was to first independently assess the effect of Rock inhibition on blastocyst formation, to bring clarity to the conflicting reports within the field. Secondly, the aim was to thoroughly characterize any observed defects in cytoskeletal, cellular-apical-basolateral polarity and Hippo-signalling, with particular respect to the subcellular localisation and functioning of the Hippo-signalling activator Amot, that had not been investigated in the context of Rock inhibition to date. Moreover, to also characterize the effects of Rock inhibition, within this context, at earlier developmental time points not previously reported. Accordingly, it can be reported that Rock inhibition during preimplantation mouse embryo development causes impaired blastocyst formation that is associated with improper apical-basolateral polarization (typified by distinct dynamics of individual polarity-related proteins) and aberrant activation of outer cell Hippo-signalling. Furthermore, this study demonstrates, for the first time, such Rock inhibition effects are mediated via the Hippo-signalling component Amot, itself mislocalised to the basolateral domains of outer cells, an effect that is also partially evident from the 16-cell stage; thus providing additional novel mechanistic insight into the regulation of differential Hippo-signalling activity in emerging early mouse embryo cell lineages.