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Msh Homeobox Genes Regulate Cadherin-Mediated Cell Adhesion and Cell–Cell Sorting

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Abstract Msx-1 and Msx-2 are two closely related homeobox genes expressed in cephalic neural crest tooth buds, the optic cup endocardial cushions, and the developing limb [Hill and Davidson, 1991; Monaghan et al., 1991; Robert et al., 1991]. These sites correspond to regions of active cell segregation and proliferation under the influence of epithelial–mesenchymal cell interactions [Brown et al., 1993; Davidson et al., 1991], suggesting that Msx-1 and Msx-2 regulate cell–cell interactions. We have investigated the potential relationship between expression of the Msh homeobox genes (Msx-1 and Msx-2) and cadherin-mediated cell adhesion and cell sorting. We report that cell lines stably expressing Msx-1 or Msx-2 differentially sort on the basis of Msh gene expression. We demonstrate in vitro that initial cell aggregation involves calcium-dependent adhesion molecules (cadherins) and that Msh genes regulate cadherin-mediated adhesion. These results support the hypothesis that Msh genes play a role in the regulation of cell–cell adhesion and provide a link between the genetic phenomena of homeobox gene expression and cellular events involved in morphogenesis, including cell sorting and proliferation. J. Cell. Biochem. 70:22-28, 1998.

Key words: Msx-1; Msx-2; homeobox; adhesion; sorting; cadherin

Homeobox genes play a central role in the process of pattern formation in the vertebrate embryo [Krumlauf, 1994]. Stem cell-mediated gene disruptions and overexpression studies using transgenic technology have demonstrated that homeobox genes regulate morphogenesis [Dolle et al., 1993; Lufkin et al., 1991; Morgan et al., 1992]. However, it remains unclear how homeobox genes affect cellular processes of patterning such as cell adhesion migration and sorting.

Msx-1 and Msx-2 are two closely related murine homeobox genes of the Msh class. Both genes are expressed in regions of active cell–cell sorting and proliferation predominantly at epithelial–mesenchymal junctions [Hill et al., 1989; Mackenzie et al., 1991; Monaghan et al., 1991; Robert et al., 1989]. The expression patterns of Msh genes in a variety of organisms, including flies, birds, frogs, and mice, suggest that Msh genes have a functional role in cells undergoing major developmental transitions.

Furthermore, a Msx-1 loss of function mutation in mice corresponds to changes in epithelial–mesenchymal interactions in the developing neural crest, craniofacial processes, otic vessels, and tooth buds [Maas and Satokata, 1994]. Mutations in the DNA binding domain of Msx-2 produce severe craniofacial abnormalities as a result of aberrant cell–cell interactions [Jabs et al., 1993].

In an effort to provide a mechanistic understanding of the functional role of Msx genes at epithelial–mesenchymal junctions, we have investigated whether Msx gene expression is sufficient to alter cell adhesion and the morphological process of cell–cell sorting. It is demonstrated that (1) expression of Msx-1 but not Msx-2 alters cadherin-mediated cell adhesion, and (2) Msx-1 and Msx-2 expression result in specific cell sorting. On the basis of these observations, we propose that Msh homeobox genes act as rapid regulators of epithelial–mesenchymal interactions by influencing the

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adhesive capacity of cadherins, which ultimately results in cell sorting.

MATERIALS AND METHODS

Cell Culture

The establishment of the cell lines used in this study has previously been described [Song et al., 1992]. The murine myoblast cell lines for this study were as follows: F31c and F31-5 (Msx-1-expressing) F3Hx8F2 (Msx-2-expressing) and the parental control line F3neo. In brief, cell lines were established by calcium phosphate transfection, followed by selection with the neomycin analogue, G418. Individual clones were isolated, and stable expression of Msx-1 or Msx-2 was confirmed using RNA-polymerase chain reaction (PCR) and ribonuclease protection.

Cell Labeling and Aggregation Assay

The aggregation assays used were based primarily on the protocol as described by Takeichi [1977], with the following modifications: monolayers of each cell type were fluorescently labeled either green or red by 45-min incubation in Dulbecco's modified essential medium (DMEM) supplemented with 5 mM 5-chloromethyl fluorescein diacetate or 5 mM (5-(and 6-)-(((chloromethyl)benzoyl)amino)tetramethylrhodamine) (Molecular Probes, Eugene, Oregon), followed by a 2-h incubation in complete media without dye. This protocol generates a covalent linkage of the dye to inner cell membranes, thus eliminating intercellular "bleeding." After labeling, cells were dissociated to single cell suspension by trituration and trypsinization in the presence of either 1 mM calcium (TC cells) 1 mM EDTA with trypsin (TE cells) or 1 mM EDTA alone (E cells) and reaggregated in the presence of 1 mM calcium. Single-cell suspensions were confirmed, visually counted, and equal numbers of homotypic labeled cells were allowed to reaggregate for 1 h at 100 rpm. After 1 h, cells were fixed in 4% paraformaldehyde/PBS and scored for percentage of green cells relative to red cells in the aggregates. Results represent the mean for at least two independent experiments.

Cell Sorting Assay

For the cell sorting assay control (F3neo), Msx-1-expressing (F31c and F31-5) and Msx-2-expressing (F3Hx8F2) cells were plated at low density (approximately 2.5×10^5 cells/100-mm plate) and allowed to reach 50–70% confluence. The cultures were prelabeled with the appropriate fluorescent tracking dye washed once with phosphate-buffered saline (PBS) and proteolytically dissociated with 1% pancreatin in PBS supplemented with 0.1% EDTA. Cell suspensions were triturated until all cell clumps were reduced to single cells. This was confirmed visually upon hemacytometer counting. Each cell type was resuspended at a density of 10^5 cells/ml in media pre-equilibrated to 5% CO_2. This suspension was transferred to sterile Eppendorf tubes sealed and allowed to incubate with gentle agitation on a Nutator unc at 37°C for 48−72 h. Preliminary experiments had shown cell survival was greater than 95% after 72 h by trypan blue dye exclusion (data not shown). Aggregates were fixed with 4% paraformaldehyde/PBS and mounted using SlowFade (Molecular Probes, Oregon) prior to microscopic analysis by epifluorescense or confocal imaging. All confocal analysis was performed using a Leica confocal microscopy station. Individual channels were scanned separately at 488 nm and 568 nm for FITC and Texas Red, respectively, to eliminate any possible signal overlap.

RESULTS

Msx-1 Alters Calcium-Dependent Aggregation

The effects of Msh gene expression on cell adhesion was tested using an in vitro aggregation assay. This assay can be used to generate by selective trypsinization cells which retain both types of cell adhesion molecules on their cell surface (E cells) cells with no CAMs (TE cells) and cells with only cadherins (TC cells). By selectively eliminating one type of CAM versus another and subsequently testing for their capacity to aggregate it is possible to determine which class of CAMs is primarily responsible for cell aggregation. This assay was carried out for control (F3neo) cells, Msx-2-expressing cells, and Msx-1-expressing cells, and the behavior of treated cells was followed using marker fluorescent dyes. The results are as follows: (1) control TC cells mixed with control TC cells formed aggregates, which equally contained both cell types (as scored by the percentage of green labeled cells in the aggregates), demonstrating that the control cell line expresses cadherins and aggregates due to the presence of these adhesion molecules (Fig. 1, bar 9); (2) control E cells mixed with control TC

cells formed aggregates composed of equal numbers of both cell types (Fig. 1, bar 8), demonstrating that control cells express both classes of CAMs. Similar results were obtained with cells expressing Msx-2 (Fig. 1, bars 4–6), showing that expression of Msx-2 has no effect on cell adhesion, as measured by this in vitro aggregation assay.

In contrast, when Msx-1-expressing cells having both class of CAMs (E cells) are mixed with cells having only cadherins (TC cells), 92% of the cells composing the aggregates are E cells (Fig. 1, bar 2). These experiments show that Msx-1 expression either changes cadherin protein levels or alters some regulatory component of the cadherin complex required for calcium-dependent cell–cell adhesion. This effect is specific to Msx-1, since Msx-2-expressing cells show no changes in calcium-dependent cell adhesion as measured by this in vitro aggregation assay (cf. Fig. 1 bar 2 vs bar 5). To confirm that Msx-1 expression affects cadherin-mediated aggregation Msx-1-expressing TC cells were mixed with Msx-1-expressing TC cells and less than 5% of the cells formed aggregates (Fig. 1, bar 3).

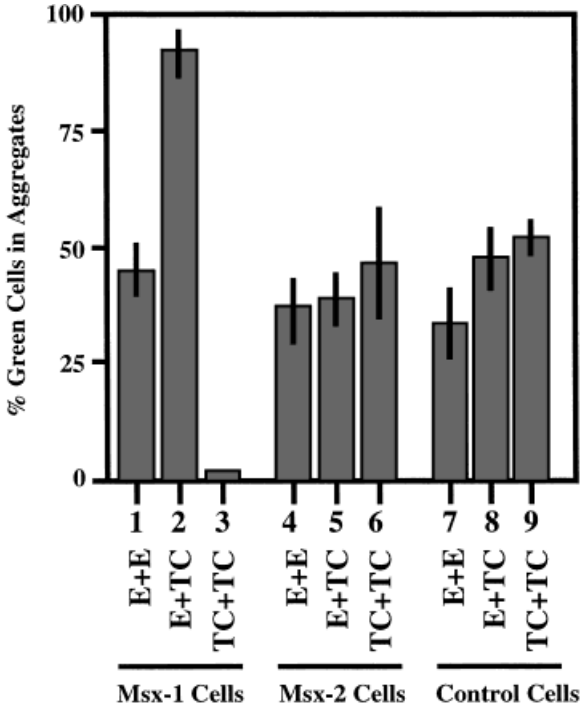

Fig. 1. Msx-1 expression decreases Ca^{2+}-dependent cell aggregation. TC cells retain intact cadherin molecules, E cells retain cadherins, and Ca^{2+}-independent adhesion molecules and TE cells have both classes removed. Control/TC $cells_{green}$ and control/TC $cells_{red}$, when mixed, have equivalent adhesivity and therefore aggregate randomly, as indicated by 53% green cells in the aggregates (bar 9). Control/E $cells_{green}$ and control/TC $cells_{red}$ also aggregate randomly (48%), indicating that aggregation is predominantly cadherin mediated (bar 8). Similarly, Msx-2 cells aggregate randomly and the aggregation is cadherin mediated (bars 4-6). In contrast, Msx-1 (+)/E $cells_{green}$, when mixed with Msx-1 (+)/TC $cells_{red}$, do not demonstrate equivalent adhesivity. TC Msx-1 (+) cells do not aggregate efficiently, resulting in aggregates composed predominantly (92%) of green E cells (bar 2) Msx-1 (+)/TC $cells_{green}$ when mixed with Msx-1 (+)/TC $cells_{red}$ form few aggregates relative to the control cells (53%) (cf. bar 3 vs bar 9), confirming that cadherin-mediated aggregation is decreased by expression of Msx-1.

Msh Gene Expression Results in Differential Cell Sorting

Because cell–cell adhesion and cadherin-mediated adhesion in particular can confer differential sorting properties to cells [Takeichi and Steinberg 1994], the effects of Msh gene expression upon cell–cell sorting were evaluated. Equal numbers of Msx-1-expressing cells were combined in suspension culture either homotypically (i.e., Msx-1 cells/Msx-1 cells) or heterotypically with the control line (i.e., Msx-1/control). To measure sorting each cell type was differentially labeled with inert fluorescent tracking dyes as described under Methods and Materials. Homotypic combinations of the Msx-1 cells resulted in a randomly mixed aggregate of cells (Fig. 2A ($Msx\text{-}1_{green}$)/($Msx\text{-}1_{red}$). A similar random mixing was observed in homotypic combinations of control cells (Fig. 2B ($control_{green}$)/($control_{red}$). In contrast, heterotypic mixtures of Msx-1 cells with control cells (Msx-1/control) showed specific sorting. The control cells formed a central core surrounded by a "shell" of Msx-1-expressing cells (Fig. 2C,D ($Msx\text{-}1_{green}$)/($control_{red}$). Similar results were seen with heterotypic mixtures of Msx-1 and Msx-2-expressing cells with Msx-2 cells forming a "shell" around a core of Msx-1 cells (Fig. 4A–D; $Msx\text{-}1_{red}$/$Msx\text{-}2_{green}$).

To quantitate cell sorting in the aggregates, all combinations of heterotypic and homotypic aggregates were prepared. Two different Msx-1-expressing lines were used for these experiments to confirm that differential sorting was not due to clonal variation. Aggregates sampled at 48 h were scored for percentage surface mixing of cells. All homotypic aggregates revealed 40–63% surface mixing (Fig. 3, bars 1–4)(50% represents random cell sorting), demonstrating that no differential sorting had occurred. In the heterotypic mixtures the percent surface mixing was 15% or less (Fig. 3, bars 5–10). These data demonstrate that sorting behavior is spe-

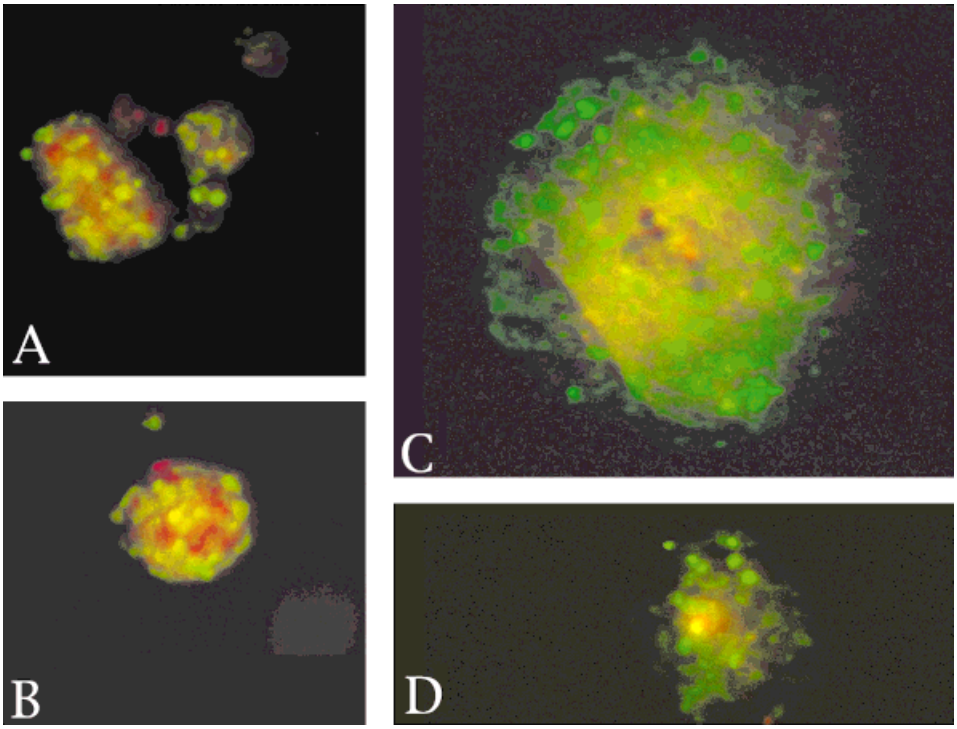

Fig. 2. Cell lines expressing Msx-1 differentially sort and segregate from control cells in suspension culture. **A:** Photomicrograph using epifluorescence of a Msx-1_{green}/Msx-1_{red} aggregate after 72 h of suspension culture shows a random aggregation of cells. Both green and red cells are expressing Msx-1. **B:** Photomicrograph of a $control_{green}$/$control_{red}$ aggregate after 72 h of suspension culture reveals a random aggregation of cells. Both green and red cells are the Msx-1 negative control line. **C,D:** Photomicrograph of a Msx-1_{green}/$control_{red}$ aggregates after 72 h of suspension culture shows a nonrandom sorting of cells (Msx-1_{green}/$control_{red}$). Representative Msx-1_{green}/$control_{red}$ (C) aggregate shows differential sorting with Msx-1 cells forming a "shell" around a core of control cells. C,D: Msx-1-expressing cells are green, and control cells are red.

cific for the Msx-1-expressing lines (see Fig. 3, bars 5 and 6). Some differences in sorting behavior do exist between the two Msx-1-expressing lines used, as nonrandom sorting is observed when the lines are mixed (Fig. 3, bar 5). However, these differences do not significantly alter the sorting behavior observed between the two Msx-1-expressing lines and either control or Msx-2 cells. In fact, when Msx-1/Msx-2 aggregates are made a central core of Msx-1 cells is formed with less than 10% surface mixing with the surrounding Msx-2 cells (Fig. 3, bars 8 and 9).

In order to assess whether the sorting phenomenon occurs throughout the aggregates, including the core, the differential sorting of several randomly chosen Msx-1/Msx-2 aggregates was analyzed in further detail using confocal imaging. Figure 4A–D is typical of the results obtained from the confocal analysis of Msx-1/Msx-2 heterotypic aggregates. For this experiment, Msx-1 cells were labeled with red fluorescent dye, and Msx-2 cells were labeled with green fluorescent dye.

Figure 4A–D represents four optical sections through a Msx-1_{red}/Msx-2_{green} aggregate. The sections begin below the external surface of the aggregate (Fig. 4A) and move progressively toward the center of the aggregate (Fig. 4D). By sequentially comparing the localization of the Msx-1 cells (red) relative to the Msx-2 cells (green), it can be seen that Msx-1 cells sort to the center of the aggregate (Fig. 4D), while Msx-2 cells preferentially sort to the exterior of the aggregate (Fig.4A). As a control, a homotypic Msx-2/Msx-2 aggregate was optically sectioned. Figure 4E is representative of the center of a typical homotypic aggregate and demonstrates that homotypic aggregates are not sorting within the aggregates relative to the heterotypic combinations (cf. Fig.4D vs 4E).

Finally, immunoblot analysis of total cellular proteins from Msx-1 cells Msx-2 cells and control cells grown as a aggregates using pan-cadherin antibodies demonstrated no significant differences in steady-state levels of classic

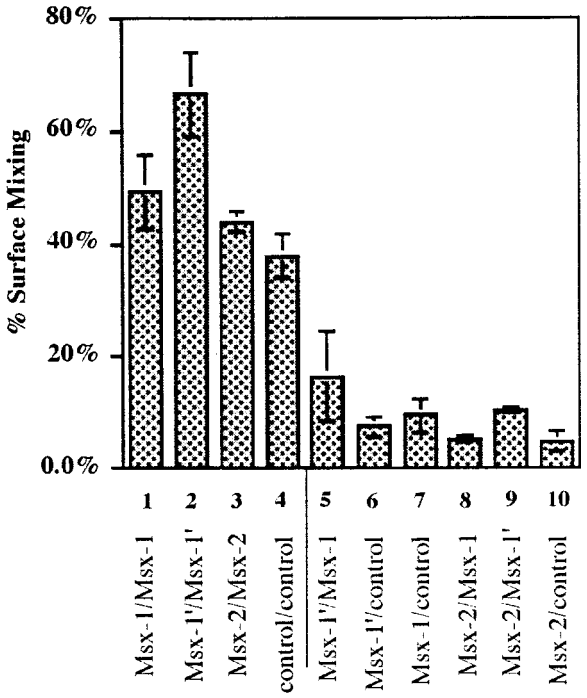

Fig. 3. Quantitation of homotypic versus heterotypic sorting. At least seven aggregates for each combination were sampled at 48 h and visually scored for percentage surface mixing of dye labeled cells. An ideal score of 50% represents random cell sorting. All homotypic aggregates (bars 1-4) show 40-63% surface mixing at 48 h, demonstrating that no differential sorting was occurring. In the heterotypic mixtures, the percentage surface mixing at 48 h was 15% or less (bars 6-10), demonstrating that differential sorting does occur in the heterotypic combinations. The unequal mixing observed for the combination of the two Msx-1-expressing lines (bar 5) probably results from differences in quantitative adhesivity between the two different cell lines but has no effect on the heterotypic sorting.

cadherins (data not shown). Additionally, immunoblot analysis of α-catenin and β-catenin indicated total catenin protein levels were equivalent in control and Msx-expressing cells (data not shown).

DISCUSSION

We have investigated the potential regulation of cell adhesion by Msh genes in an effort to establish whether homeobox gene expression is sufficient to alter the physical process of cell adhesion and the morphological process of cell sorting. Using an in vitro cell aggregation assay, it can be demonstrated that Msx-1 significantly decreases calcium-dependent cell aggregation. Msx-2 a closely related family member does not change calcium-dependent aggregation under the conditions of the assay used. This differential effect of the two homeobox genes, which share a nearly identical DNA binding domain would suggest that these two genes have specific cellular functions. The aggregation assays also demonstrate that neither Msh gene alters calcium independent aggregation within the context of the described experimental cell system.

Since changes in calcium-dependent adhesion have been demonstrated to alter cell sorting [Nose et al., 1988], we attempted to determine whether Msh gene expression was sufficient to alter differential cell sorting. Consistent with results from our in vitro aggregation assays Msh gene expression is sufficient to confer differential cell sorting properties to aggregated cells in culture. The Msx-1-mediated decrease in calcium-dependent adhesion seen in our in vitro aggregation assays is consistent with the idea that shifts in the cellular localization of cadherins may act as a rapid regulatory mechanism during cell–cell sorting. This mechanism could function by maintaining intracellular pools of cadherins while simultaneously blocking their transport to the cell surface. In this scenario, cells have the ability to rapidly shift assembled cadherin complex to the cell surface. This mechanism is consistent with the known requirements for correct assembly of the α-catenin/β-catenin/plakoglobin complex of proteins with the cadherins in MDCK epithelial cells [Hinck et al., 1994a].

Finally, the observation that total levels protein levels of cadherins and catenins are not altered by Msh gene expression does not rule out the possibility of regulation (i.e., phosphorylation) or of modification of the stability of protein–protein interactions of catenins with cadherins as a result of Msh gene expression. The possibility of additional regulatory mechanisms involving catenins is supported by experiments indicating that the wingless homologue Wnt-1 can regulate cell–cell adhesion by stabilizing the cadherin/α-catenin complex without altering the expression or binding of β-catenin [Hinck et al. 1994b].

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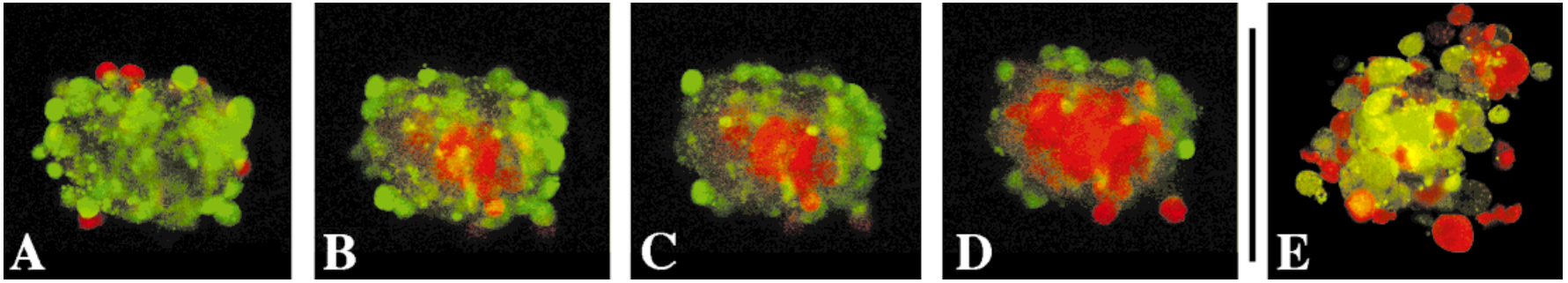

Fig. 4. Confocal optical sections of a Msx-1green/Msx-2red aggregate confirms differential sorting results from Msh gene expression. **A–D:** A Msx-1_{red}/Msx-2_{green} aggregate after 72 h of suspension culture was optically sectioned at 1 µm, using a Lieca TCS 4D laser scanning confocal microscope starting above the equatorial plane of the aggregate and moving inward toward the equator of the aggregate. A: The first section with subsequent sections at 5-µm intervals (moving equatorially) Msx-1-expressing cells are red, and Msx-2-expressing cells are green. Scale bar = 15 µm. **E:** Equatorial optical section of a Msx-2_{green}/Msx-2_{red} aggregate at 72 h shows no differential sorting. This section is representative of all homotypic aggregates.

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