Chromosome instability drives phenotypic switching to metastasis

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Chromosome instability (CIN) is the most striking feature of human cancers. However, how CIN drives tumor progression to metastasis remains elusive. Here we studied the role of chromosome content changes in generating the phenotypic dynamics that are required for metastasis. We isolated epithelial and mesenchymal clones from human carcinoma cell lines and showed that the epithelial clones were able to generate mesenchymal variants, which had the potential to further produce epithelial revertants autonomously. The successive acquisition of invasive mesenchymal and then epithelial phenotypes recapitulated the steps in tumor progression to metastasis. Importantly, the generation of mesenchymal variants from clonal epithelial populations was associated with subtle changes in chromosome content, which altered the chromosome transcriptome and influenced the expression of genes encoding intercellular junction (IJ) proteins, whereas the loss of chromosome 10p, which harbors the ZEB1 gene, was frequently detected in epithelial variants generated from mesenchymal clones. Knocking down these IJ genes in epithelial cells induced a mesenchymal phenotype, whereas knocking down the ZEB1 gene in mesenchymal cells induced an epithelial phenotype, demonstrating a causal role of chromosome content changes in phenotypic determination. Thus, our studies suggest a paradigm of tumor metastasis: primary epithelial carcinoma cells that lose chromosomes harboring IJ genes acquire an invasive mesenchymal phenotype, and subsequent chromosome content changes such as loss of 10p in disseminated mesenchymal cells generate epithelial variants, which can be selected for to generate epithelial tumors during metastatic colonization.

Significance

Chromosome instability and its resulting karyotypic heterogeneity make up one of the most striking characteristics of human cancers. Yet whether chromosome loss or gain drives tumor progression to metastasis remains unknown. Here we show that clonal populations of epithelial cells spontaneously generate mesenchymal variants. These variants have potential for reverting to an epithelial phenotype. Importantly, we show that the successive phenotypic variants selectively eliminate or acquire chromosome segments that harbor genes encoding intercellular junctional proteins and their regulators. Thus, tumor metastasis can be a clonal process driven by chromosome instability.

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Spectral karyotyping (SKY) analysis indicated that both epithelial and mesenchymal clones shared several derivative chromosomes with their parental OV5-P cells, which further proved their common origin (SI Appendix, Table S1). Although the karyotypes among the three epithelial clones or among the mesenchymal clones were similar, numerous chromosomal changes were observed between the two different types. Although the epithelial clones exhibited a highly homogeneous phenotype, rare mesenchymal-like cells were observed during clonal expansion. We isolated three of these phenotypic variants from the OV5-E1 clone (Fig. 1G) on the basis of their grape-like morphology in 3D culture. The variants were termed OV5-E1M1, OV5-E1M2, and OV5-E1M3, with “OV5-E1” referring their origin and “M” to their acquired mesenchymal phenotypes. All the mesenchymal variants grew in a scattered pattern with the loss of E-cadherin in immunofluorescent staining (Fig. 1H), as well as a gain of ZEB1 in Western blot analysis (Fig. 1I). To evaluate the role of CIN in generating these mesenchymal variants, we used fluorescence in situ hybridization (FISH) analysis with a probe covering the E-cadherin gene (CDH1). All the epithelial clones (OV5-E1, OV5-E2, and OV5-E3) contained three copies of 16q, whereas two of the three M clones (OV5-M1 and OV5-M2) contained two copies of that arm (Fig. 1J and SI Appendix, Table S3). Importantly, two of the three mesenchymal variants (E1M1 and E1M3) that derived from OV5-E1 showed a decreased copy number of CDH1, suggesting a role of 16q loss in the down-regulation of E-cadherin and the generation of mesenchymal variants.
Although the mesenchymal clones exhibited nearly homogeneous morphology when cultured in Petri dishes, small epithelial-cell-like clusters were occasionally noticed. We reasoned that the single-cell-derived mesenchymal cell population might have generated epithelial variants during cell division. Taking advantage of the differential sensitivity to trypsinization between epithelial and mesenchymal cells, we enriched the epithelial variants by continuously culturing cells to confluence and then removing the mesenchymal cells with controlled trypsinization. Thus, we obtained two cell populations: OV5-E1M1E from OV5-E1M1 and OV5-M2E from OV5-M2 (Fig. 2A). Epithelial cells were dominant in both populations, as shown by the dramatic down-regulation of ZEB1 and up-regulation of E-cadherin (Fig. 2B and C). FISH analysis with probes covering CDH1 gene on 16q indicated that 100% of OV5-E1M1E and OV5-M2E cells contained two copies of 16q, which was the same as their parental cells. This result excluded the possibility that the up-regulation of E-cadherin in the epithelial revertants was through regain of 16q (SI Appendix, Table S3).

The ZEB1 gene, located at 10p11.2, codes for a protein that is a transcriptional repressor of E-cadherin (21, 22). Knocking-down of ZEB1 with siRNA induced an epithelial morphology with a dramatic increase of E-cadherin protein in both OV5-E1M1 and OV5-M2 cells, indicating that down-regulation of ZEB1 was sufficient to drive the epithelial phenotype (Fig. 2D and E). Therefore, we tested whether the generation of epithelial phenotype was by loss of 10p. FISH analysis showed that 92.5% of OV5-E1M1E cells and 75% of OV5-M2E cells contained one copy of 10p, which is one copy less than their parental cells (Fig. 2F and SI Appendix, Table S4). SKY assay revealed that OV5-E1M1E cells contained two copies of full chromosome 10, whereas OV5-E1M1E cells had one copy of full chr10 and one copy of a derivative chromosome that contained only 10q (Fig. 2G). In half of the cases, the derivative chromosome was i(10)(q10), which resulted from head-to-head fusion of two 10q’s. In the other half, a copy of 10q was joined to various other chromosome segments including 22q, 20q, 3p/pter-q21), 19p, der(2;15)(p10;q10), and 14q (Fig. 2G). OV5-M2E also lost one copy of 10p, but 10q invariably translocated to 7q in all 20 of the cells analyzed. The fact that both revertants lost 10p, but reserved 10q in various different ways, suggested that genes on 10p were pivotal for maintaining a mesenchymal phenotype, whereas the genes on 10q might be essential for an epithelial phenotype or cell survival. Therefore, chromosome breakage and rearrangement makes it

![Image of Figure 2](https://example.com/image2)

Fig. 2. Loss of 10p in generating epithelial revertants from clonal mesenchymal cells. (A) Morphology of epithelial revertants cultured in a Petri dish. (B) Real-time RT-PCR and/or Western blot assay showing loss of ZEB1 and gain of E-cadherin in epithelial revertants. (C) Membranous E-cadherin staining in epithelial revertants. (D) Epithelial morphology in OV5-E1M1 and OV5-M2 cells treated with siRNA to ZEB1 (Zeb1-si1). (E) Western blotting showing E-cadherin induction after ZEB1 knock-down with siRNA in OV5-E1M1 and OV5-M2 cells. Cells were treated with control siRNA (Control) or two siRNA against ZEB1 (Zeb1-si1 and Zeb1-si2). (F) FISH analysis for copy number of the ZEB1 gene on 10p11.2. (G) SKY assay on epithelial revertants and their parental cells; representative karyotypes are shown. Variations of chr10 from 20 metaphases are depicted in red frames below the karyotypes. Numbers in parentheses indicate the incidences of the above chr10 pattern in 20 karyotypes. Metaphase FISH was performed to confirm head-to-head fusion of two 10q’s in OV5-E1M1E cells and the identities of der containing chr10 in OV5-M2E cells (Bottom red frame). FISH probe on 10q25.3 was labeled with red color. Probe on 10p11.2 for OV5-E1M1E and 10p15.1 for OV5-M2E were labeled with green.
possible to eliminate a smaller chromosomal region containing target gene or genes through chromosome loss. Importantly, all the OV5-E1M1E cells retained a copy of der(21)(1;21;11;13)(p11;p11.1q22;q13q25;q11), which was unique to OV5-E1M1E. Similarly, OV5-M2E and OV5-M2 shared unique i(8)(q10) and der(16)(13;16)(q22;q11.2) derivative chromosomes and presented almost identical karyotypes except for chromosomal changes that involved chr10 and chr7. Together, these results demonstrated that OV5-E1M1E and OV5-M2E cells generated epithelial revertants by selectively eliminating 10p.

To seek another paradigm for CIN determination of the metastatic phenotype, we studied DU145, a prostate carcinoma cell line that has a heterogeneous phenotype when cultured in Petri dishes (SI Appendix, Fig. S2). Parental DU145 cells formed hollow acinar structures or loose cell aggregates when cultured in 3D Matrigel. In the presence of hepatocyte growth factor/scatter factor (HGF/SF), the loose cell aggregates formed branching structures, which are more easily distinguishable from acini. We therefore isolated an acini-forming clone (DU-E) and a branch-forming clone (DU-M) on the basis of their morphology in 3D culture in the presence of HGF/SF. From the DU-E population, we further isolated three branching clones (DU-EMn) that maintained a branching phenotype after multiple cell passages (Fig. 3D). DU-U displayed a high level of E-cadherin as well as other IJ proteins, which are markers for an epithelial phenotype (Fig. 3B and C and SI Appendix, Fig. S2). These epithelial markers were decreased dramatically in DU-M and DU-EMn cells. Both DU-M and DU-EMn clones were more invasive than DU-E cells (Fig. 3D).

A FISH assay indicated that the copy number of 16q22 did not decrease in DU-M or DU-EM cells (SI Appendix, Table S5). To determine whether changes in chromosomes harboring other IJ genes could be involved in phenotype determination, we performed comparative genomic hybridization on DU-M and DU-EMn cells, using DU-E cells as a reference (Fig. 3E). Significant decreases of 1q, 4p, 6p, 14q, and 18p/q were detected in DU-EMn cells. Comparative genomics microarray analysis indicated that the chromosome content changes closely correlated with chromosome transcriptome changes (Fig. 3E), which was consistent with our previous report (23). These transcriptome changes were barely detected in DU-EE clones, suggesting that the changes were relevant to a mesenchymal phenotype. A SKY assay indicated that decreases

Fig. 3. Phenotypic characterizations of clones from DU145 cells (A) Branching morphogenesis assay. Cells cultured in 3D were stained with calcein-AM and observed by confocal microscopy. (B) Western blotting showing the down-regulation of intercellular junction proteins in cells with a mesenchymal phenotype. (C) Immunofluorescence staining showing the loss of adherent junctions and tight junctions in mesenchymal clones; the counterstain was Hoechst 33342. (D) Invasion results in Matrigel chamber assay. (E) Comparative genomic hybridization and comparative genomics microarray analysis showing chromosome dosage and chromosome transcriptome changes (log2 transformed chromosome transcriptome ratio between indicated clones and DU-E); blue, decrease; red, increase. (F) Chromosome content changes in DU-E, DU-M, and DU-EMi3 cells. The chromosomes or der chromosomes that contain the indicated chromosome content are listed. Loss of 1q, 4p, 6p, 14q, and 18p/q plus gain of 3q in mesenchymal clones resulted from complicated chromosome changes. (G) Knocking down of intercellular junction proteins in DU-E cells induced mesenchymal phenotype. Cells were treated with a control siRNA (NC), or a combination of siRNAs to CGN, DSP, DSC2, and DSG2 (IJ). Down-regulation of E-cadherin protein was detected with Western blotting. (H) Immunofluorescence staining showing the loss of E-cadherin and cytoplasmic translocation of ZO-1 at 48 h after transfection with siRNA to CGN, DSP, DSC2, and DSG2 (IJ).
of 1q, 4p, 6p, 14q, and 18p/q resulted from the loss of der(1)(1;4)(p11;p11);der(6;16)(q11;p11.2) and der(18)(14;18)(q13;q12), which was observed in DU-E (but not in DU-EMn) cells (Fig. 3F and SI Appendix, Tables S6–S9 for detailed calculation of chromosome content changes). Nonetheless, DU-E and DU-EMn cells shared der(13)(1;8;13;1;19), der(8)(7;8)(p11.1;p11.1), and del(15), which were not found in the DU-M and parental DU145 cells, indicating the lineage relation between DU-E and DU-EMn cells (SI Appendix, Table S8). Chromosome copy number changes dramatically influenced the expression of their resident genes: 18 of the 19 significantly altered genes changed expression in accord with the change in their host chromosome (SI Appendix, Tables S10 and S11). Down-regulation of six IJ genes, including CDH1 (E-cadherin), CGN (cingulin), CLDN7 (claudin7), DSP (desmoplakin), DSC2 (desmocollin2), and DSG2 (desmoglein2), were found in DU-M and DU-EMn clones (Fig. 3B and SI Appendix, Fig. S3 and Table S10). Four of these genes were on chromosomes having copy number decreases (SI Appendix, Table S12). Although the copy number of the CDH1 gene did not decrease, vigorous rearrangement occurred between 10q and various other chromosomes (SI Appendix, Fig. S4). To confirm that the chromosome-loss-associated down-regulation of IJ proteins contributed to the generation of DU-EMn clones from DU-E, we knocked down the expression of CGN, DSP, DSC2, and DSG2 in DU-E, using a combination of siRNAs to each gene (Fig. 3G). Cells treated with siRNA exhibited disrupted tight junctions and adherens junctions, as indicated by the cytoplasmic location of ZO1 and the decrease of E-cadherin protein (Fig. 3H). These results suggest the loss of chromosomes harboring IJ genes can be a mechanism for generating a mesenchymal phenotype. Unlike with OV5-P cells, the in vitro selection of epithelial variants from DU145 mesenchymal clones was not feasible, partly
because of the similar sensitivity to trypsinization between epithelial and mesenchymal clones. The fact that metastatic tumors frequently have an epithelial phenotype suggests epithelial cells may have a growth advantage. We tested whether the isolated DU-M and DU-EM1 cells switched to an epithelial phenotype through tumorigenesis when inoculated s.c. into SCID mice. From tumor xenografts of these two lines, we established the cell lines DU-MET1 and DU-EM1ET1 (Fig. 4A). Both cells exhibited a predominantly epithelial phenotype, as judged by their morphobiology in 3D culture (Fig. 4B) and their expression of E-cadherin (Fig. 4C). SKY analysis revealed nearly identical karyotypes between DU-M and DU-EM1ET1 (Fig. 4D and Table S13 and S14). However, all DU-EM1ET1 cells contained three copies of 5q, whereas chr5 alterations in DU-M were highly diverse, showing seven different karyotypic patterns in 10 cells (Fig. 4D), and eight of these 10 cells contained one or two copies of 5q. We further performed FISH analysis on a mixture of eight cell lines established from tumor xenografts of DU-M (Table S15) and found that 79.5% of cells contained three or more copies of 5q, confirming that selection of cells with more copies of 5q was a common event during tumorigenesis of DU-M.

Transcriptome analysis identified 14 genes on 5q that were up-regulated in DU-EM1ET1 cells and no genes that were down-regulated (Table S15). The OCLN gene, which encodes occludin, a major component of tight junctions, was the only 5q gene. The gain in 5q was also associated with the up-regulation of kidney and brain protein (KIBRA) (5q34), an activator of SAV1 that is part of the SHW inhibitor complex of β-catenin (24, 25). Also up-regulated was CXXC finger protein 5 (CXXC5) (5q31.2), which inhibits β-catenin (26). Because the role of β-catenin in cell invasion is well established, up-regulation of KIBRA and CXXC5 might also contribute to the epithelial phenotype of DU-EM1ET1. We therefore examined the effects of siRNA knock-down of OCLN, KIBRA, and CXXC5 on the phenotype of DU-EM1ET1 cells. Our results showed that knocking-down of OCLN triggered a significant down-regulation of E-cadherin, and that this was true for KIBRA and CXXC5, to a lesser extent (Fig. 4E).

The DU-EM1 clone and its derivative DU-EM1ET1 showed a major difference in chr10: DU-EM1 cells possessed two copies of chr10 per metaphase, whereas DU-EM1ET1 contained one full chr10 and one copy of der(10;16)(p11.2; p11.2) (Fig. 4F and Table S16). Because of der(10;16)(p11.2; p11.2), DU-EM1ET1 lost one copy of 10p relative to DU-EM1. Expression array analysis revealed that two genes in the 10p region were significantly altered in DU-EM1ET1 relative to DU-EM1: ZEB1 and SFBMT2 were both down-regulated (Table S17), correlating with the reduction in 10p chromosome dosage. To confirm that 10p loss in DU-EM1ET1 contributed to an epithelial phenotype, we treated DU-EM1 cells with siRNA against ZEB1 and found a dramatic increase of E-cadherin protein (Fig. 4G). These results showed that, as in OV5 cells, 10p loss also played a causal role in generating the epithelial phenotype of DU-EM1ET1 cells.

Taken together, our results demonstrate that tumor cells undergo constant structural and numerical chromosomal changes that lead to karyotype diversity in a clonal cell population. In epithelial cells, the loss of chromosome segments harboring genes encoding IJ proteins results in down-regulation of IJ proteins and the generation of mesenchymal variants that facilitate metastasis and chemoresistance. Subsequently, loss of chromosome fragments carrying transcriptional repressor of IJ proteins, such as ZEB1 in disseminated mesenchymal cells, leads to generation of epithelial variants that can be selected for in appropriate environments to generate metastatic colonization. Thus, the sequential phenotypic changes during tumor metastasis can be a process of Darwinian selection of phenotypic variants generated by chromosome instability.

Materials and Methods
Cell Lines and Reagents. OVCAR5 and DU145 cells were obtained from the National Cancer Institute. The authenticity of the cell lines was proved by karyotyping. Unless specifically indicated, cells were cultured in RPMI medium supplemented with 5% (vol/vol) FBS (Invitrogen). Mouse anti-E-cadherin (HECD-1), rabbit anti-desmoglein, mouse anti-desmoglein (3G12), and mouse anti-β-actin (AC-15) antibodies were purchased from Abcam. Mouse anti-γ-tubulin was from Sigma-Aldrich. Mouse anti-desmoscinolipin2 and rabbit anti-ZO-1 were from Invitrogen. Antibody against ZEB1 (H-102), as well as horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit secondary antibodies, were from Santa Cruz Biotechnology.

3D Cell Culture and Phenotype-Based Subcloning. Cells were collected by trypsinization and suspended in culture medium at a concentration of 5,000 cells/mL. Fifty microliters of cell suspension was mixed with an equal volume of Matrigel (BD Bioscience) and placed into 96-well plates. After 30 min incubation at 37°C, cells were grown on regular culture medium for 5–7 d to allow the formation of acini or grape-like aggregates. For isolation of epithelial or mesenchymal clones from OVCAR-5 cells, suspended in culture medium at low density were mixed with equal volume of Matrigel and seeded into 6-μm Petri dishes. For isolation of acini or branching structures from DU145 cells, cultured in 3D in the presence of HGF/SF (100 ng/mL), acini and branching or grape-like aggregates growing in 3D Matrigel were selected and isolated. Cells derived from individual acini or aggregates were diluted to 5 cells/mL and seeded in 96-well plates. Wells containing single cells were marked and expanded to obtain clones.

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