Polyploidization of murine mesenchymal cells is associated with suppression of the long non-coding RNA H19 and reduced tumorigenicity

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Abstract

Mesenchymal stromal cells (MSCs) are used extensively in clinical trials; however, the potential for malignant transformation of MSCs has been raised. We examined the genomic stability versus the tumor forming capacity of multiple mouse MSCs. Murine MSCs have been shown to be less stable and more prone to malignant transformation than their human counterparts. A large series of independently isolated MSC populations exhibited low tumorigenic potential under syngeneic conditions, which increased in immune-compromised animals. Unexpectedly, higher ploidy correlated with reduced tumor forming capacity. Furthermore, in both cultured MSCs and primary hepatocytes, polyploidization was associated with a dramatic decrease in the expression of the long non-coding RNA H19. Direct knockdown of H19 expression in diploid cells resulted in acquisition of polyploid cell traits. Moreover, artificial tetraploidization of diploid cancer cells led to a reduction of H19 levels, as well as to an attenuation of the tumorigenic potential. Polyploidy might therefore serve as a protective mechanism aimed at reducing malignant transformation through the involvement of the H19 regulatory long non-coding RNA.
Introduction

Bone marrow stromal cells were first thought to serve as a tissue scaffold for hemopoietic cells (1). Further studies revealed their regulatory role in protecting hemopoietic stem cells from extinction through over-differentiation (2). Eventually, these cells were designated as mesenchymal stromal/stem cells (MSCs) to denote their capacity to differentiate into a spectrum of cell types (3). MSCs have potential cell therapy uses (3-5), however, the possible susceptibility of MSCs to malignant transformation puts their application in question. It appears that culturing of murine MSCs results in the accumulation of chromosomal aberrations, such as aneuploidy and polyploidy, which might lead to the acquisition of tumorigenic properties (6, 7). Though human MSCs were initially suspected of being prone to spontaneous malignant transformation in culture (8, 9), such claims were later dismissed (10-12). In fact, human MSCs generally undergo growth arrest after a few passages in culture, necessitating the use of mouse MSCs as a model. The possible tumorigenic potential of MSCs remains at the moment an open question.

Many tumor types contain cells with abnormal amounts of DNA, e.g., aneuploid and polyploid cells (13-16). It is believed that polyploidy permits cells to acquire genetic defects, in a process termed 'chromosome instability' (CIN) (17-19). Such chromosomally unstable cells might eventually lead to tumor formation (20, 21). However, the fact that polyploid cells are also present in normal tissues, such as in the liver (22), and heart (23), suggests that polyploidy might have important physiological roles. A recent study following polyploid liver cells in vivo showed that normal hepatocytes have properties similar to that of cancer cells, as they exhibit dynamic changes in their genome resulting in vast polyploidy and aneuploidy (24). It is suggested that such dramatic genomic abnormalities allows hepatocytes to adapt to the genotoxic stress present in the liver tissue.

In the current study, a collection of individually isolated mouse MSCs were examined for their ploidy and corresponding tumorigenic potential. Polyploid MSCs were found to be by far less tumorigenic than their diploid counterparts. We therefore aimed at unraveling the mechanism by which these polyploid cells maintain a non-tumorigenic state. Genomic analysis implicated the non-coding RNA H19, a molecule shown to have both tumor-promoting (25, 26) and tumor-
suppressing (27) activities, as the major molecular marker that distinguishes between diploid and polyploid MSCs and predicts their corresponding tumor forming capacity.
Materials and Methods

Mice

The Weizmann IACUC approved all animal experiments. C57Bl, BALBc, SJL, and NOD.SCID mice were purchased from the Harlan Laboratories. NOD.SCID and NOD.SCIDβ2-microglobulin knockout mice were gifts of Prof. Tsvee Lapidot (WIS, Israel) and Dr. Nadir Askenazy (Schneider Hospital, Israel).

Cell culture

MSCs were grown in murine MesenCult Basal Media and supplement (Stem Cell Technologies), or in DMEM (Invitrogen) with 10% FCS (Biological industries), added with 60 μg/mL penicillin and 100 μg/mL streptomycin.

Derivation of MSC populations and clones

MSC populations were derived as previously described (28, 29). Briefly, cells were flushed from femurs and tibias using MSC medium and cultured until sufficient amounts of cells were obtained. For clonal isolation, MSCs at passages 5-7 were seeded in 96-well plates (Falcon) at a concentration of 0.2 cells/well and grown in MSC medium. Only wells containing single colonies were used for further experiments.

Cytogenetic analysis

Chromosome preparations and SKY analysis were acquired as previously described (30) and 25-75 mitoses were analyzed for each cell line.

DNA content estimation using flow cytometry

Cells were fixed with 70% ethanol/PBS, treated with 20μl RNaseA 10mg/ml (Sigma), and stained with 20μl propidium iodide 2.5mg/ml (Sigma). Labeled cells were analyzed using a
FACScan flow cytometer (Becton Dickinson Immunocytometry Systems). Fresh splenocytes were used as control diploid cells.

**In vivo tumorigenic potential estimation**

Cells were injected subcutaneously (s.c., 1x10^6, 3x10^6 or 5x10^6 cells) or intravenously (i.v., 0.5x10^6 cells) into 12-14 weeks old NOD.SCID, NOD.SCIDβ2 or syngeneic mice. Tumor formation was inspected for six months. The doubling time of the tumor was calculated as follows: \( T_d = \frac{\log(2) \times (T_2 - T_1)}{\log(V_2/V_1)} \) (\( T_d \): doubling time, \( T_2 - T_1 \): time between measurements, \( V_{1,2} \): the volume of the tumor at time points 1 and 2). Sections were prepared from sample tissues and tumor biopsies, stained with hematoxylin-eosin staining, and photographed using an Olympus IX71 equipped with a DP51 camera.

**Real time PCR**

RNA was extracted using Tri-Reagent (MRC) and cDNA was prepared using M-MLV Reverse Transcriptase enzyme (Promega) according to manufacturer protocols. All samples were treated with TURBO DNA-free Kit (Ambion). Real time PCR was done using Platinum® SYBR® Green qPCR SuperMix (invetrogen) and processed using ABI 7300 (Applied Biosystems). Primers are listed in supplementary table 1. cDNA of normal and immortalized primary human fibroblasts was provided by Prof. Varda Rotter (WIS).

**Microarray analysis**

Experiment was done using 100ng of total RNA in a DNA Mouse Gene ST 1.0 microarray (Arrymetrix®) at the Genomic Technologies Unit (Biological Services, WIS). Analysis was done at the Bioinformatics Unit (Biological Services, WIS) using Partek® Genomics Suite (Partek Inc., St. Louis, Missouri 63141). Data was preprocessed and normalized using the RMA (Robust Multichip Average) algorithm. Gene enrichments were found using Ingenuity Systems Pathway Analysis, GSEA, and MSigDB softwares. Microarray data is available at the GEO website, accession number GSE39410.
Immunoblot analysis
The following antibodies were used: p53 (Santa Cruz), AuroraB (BD Biosciences), GAPDH (Sigma), c-Myc (Cell signaling). Densitometry was done using ImageJ.

Isolation of hepatocytes with defined ploidy
Primary hepatocytes (from 10-14 days suckling mice and 8-10 weeks adult BALBc mice) were isolated by two-step perfusion (24). Cells were incubated with 15μg/ml Hoechst 33342 (Invitrogen), and sorted using a SORP FACSARiaII cell sorter (Becton Dickinson).

Generation of tetraploid cells
Tetraploidization was done as previously reported (31). Briefly, cells were treated with 1mM hydroxyurea (Sigma) followed by 5mM sodium butyrate (Sigma). Control treatment included 24 hours incubation with fresh medium before sodium butyrate treatment.

H19 knock-down
Procedure was done according to the Amaxa (Lonza) manual using 2μg H19 siRNA (Stealth RNAi™ siRNA, Invitrogen. Sequences: CUUUCUGUCACAUUGACCACACCUG or UCUGAUUGCAGCAUCUUUCUGAUUC). For control, 2μg of Stealth RNAi™ siRNA Negative Control Med GC (Invitrogen) was used.

Seeding efficiency
A total of 500 cells were seeded in 100 mm plates (Falcon), incubated for nine days, and stained with Giemsa/May-Grunwald (Sigma). Seeding efficiency was calculated by dividing the number of colonies counted with the number of cells seeded.
**Doubling time**

A total of 5,000-20,000 cells were seeded in 60 mm plates (Falcon), collected upon reaching ~50% confluence and counted. Doubling time was calculated using the following formula:

\[ T_d = \frac{(t_2 - t_1) \times \log(2)}{\log(q_2/q_1)} \]

where: \( t_2 - t_1 \) equals the time of incubation, \( q_1 \) is the initial amount of cells and \( q_2 \) is the final amount of the cells.

**UV sensitivity**

Cells were irradiated at UV-C (254 nm) using a low-pressure mercury lamp (TUV 15w G15T8, Philips). The number of cells one day after irradiation was divided by the number of cells in non-irradiated cultures to evaluate UV sensitivity.

**Immunofluorescence**

Cells on cover slips were fixed with ice-cold methanol, blocked and incubated with a γ-tubulin (Sigma) or AuroraB (Cell signaling) antibodies, followed by incubation with Cy3 secondary antibody (Jackson). Cover slips were mounted and counterstained using 10μl of DAPI II (Vysis). Photographs were taken using a Zeiss Axio Imager Z1 microscope (Carl Zeiss).

**Statistics**

Significance of Kaplan-Meier comparisons was determined using logrank test. All statistical analysis was done using SPSS (IBM) or Medcalc statistical software.
Results

**Polyploid MSCs maintain a non-tumorigenic state.** We analyzed cytogenetically four independently isolated MSC populations and one clone, using spectral karyotyping (SKY) (Fig. 1A). The presence of structural chromosomal aberrations on single or paired homologous chromosomes in non-diploid cells was detected (Fig. 1A and supplementary table 2). This made it possible to follow the order of numerical and structural changes in chromosomes (Fig. 1B). Three of the cell populations studied had a near diploid-tetraploid chromosomal content (MSC7, 8, and 9). One population (MSC20) and one clone (Clone1) were near-tetraploid. The proportion of each sub-population was examined by flow cytometry (Fig. 1C). The tetraploidy of MSC20 and Clone1 was stable during long-term culture. In contrast, MSCs 7, 8 and 9 were unstable; at different passages pure diploid genotypes were observed and designated accordingly (MSCs 1, 2, and 3 correspondingly). In order to explore the extent of polyploidy of MSCs in culture, we derived a total of 28 independent MSC populations, which showed variable ploidy (Fig. 1D). Most of these populations were polyploid or contained a polyploid sub-population, and only a few MSCs remained diploid (6/28 populations). We also derived 47 clonal isolates from three of the diploid-tetraploid populations (MSC7, 8 and 9); none of them were found to be diploid, suggesting that the diploid cells tend to become polyploid. Under syngeneic conditions most MSCs did not form tumors, and therefore immune-compromised mice were used to more critically assess tumorigenicity (Fig. 2). The tetraploid cells, MSC20 and Clone1, were non-tumorigenic at all passages. In contrast, MSCs 7, 8 and 9 were capable of tumor formation but showed limited tumorigenic potential, compared to their diploid counterparts MSCs 1, 2 and 3 respectively (Fig. 2A and supplementary table 3). Typically, MSC1 formed fibrosarcomas, whereas MSCs 2 and 3 formed fibro-osteosarcomas (Fig. 2B). Surprisingly, the tetraploidy of MSCs predicted lower rather than higher tumorigenicity. In order to validate this observation, the tumorigenic potential of the various MSC populations and clones was evaluated and is summarized in Fig. 2C (The tumorigenicity of individual cells is shown in Supplementary tables 4 and 5). We found that the tumorigenicity of MSCs decreased significantly with increased ploidy. Injection of diploid MSCs resulted in the highest tumorigenic incidence, followed by mixed diploid-tetraploid MSCs (>3 fold reduction), pure tetraploid MSCs (>13 fold reduction), and finally over-tetraploid MSCs, which were completely devoid of tumor forming capacity. Tumors derived from MSC7, MSC8 and MSC9 were analyzed cytogenetically (supplementary
Fig. S1). Apparently, all the tumors formed harbored cytogenetical aberrations identical to those first identified in the cultured cells before their introduction into animals. A tumor formed by MSC7 contained 2xder(19)t(7;19), MSC8 tumor contained der(4)t(4;18), and MSC9 tumor contained an additional chromosome 6 (+6). Some new aberrations have accumulated in vivo, however, most were non-clonal (detected in single cells), and overall were not common. Importantly, the tumors formed in vivo originated from the diploid rather than from the tetraploid subpopulations of MSC8 and MSC9. This is evident from the iso-chromosome 3 i(3), and der(17)t(3;17) found in all of the MSC8 and MSC9 (respectively) tumor diploid cells, which doubled in the tetraploid tumors. The fact that the MSC7 tumor only harbors two der(19)t(7;19) translocations, and not four, also suggests that this tumor was formed from the MSC7 subpopulations with lower ploidy. This data indicate that the cells responsible for tumor formation in vivo were diploid and that the polyploid cells within the tumors emerged in situ.

**Expression of the non-coding RNA H19 distinguishes between diploid and polyploid cultured MSCs and uncultured liver cells.** To identify a possible gene expression profile, which might explain the difference in the tumorigenic potential among MSCs, a DNA microarray analysis comparing three diploid with three tetraploid cells was performed (Fig. S2). From a total of 136 differentially expressed genes (p-value<0.05, fold change>2), 40 were up-regulated in diploid cells, and 96 were up-regulated in tetraploid cells (Fig. S2A). The top ten expressed genes in diploid and tetraploid MSCs are shown in Figures S2B and S2C respectively. H19, a paternally imprinted non-coding RNA molecule (32, 33), showed the highest increase in the diploid cells with a fold change of 35.9 and a p-value of 0.0018 (Fig. S2B). In addition, the function of major transcriptional regulators was different between diploid and tetraploid MSCs (Fig. S2D). Apparently, c-Myc was more active and p53 was less active in diploid compared to tetraploid MSCs (Figures S2E and S2F). Finally, many cell cycle related genes were up-regulated in diploid compared to tetraploid MSCs (Fig. S2G).

Differences in H19 expression were also evident in non-mesenchymal primary mouse liver cells (Fig. 3). We confirmed previous data showing that tetraploid cells arise in the adult mouse liver (Fig. 3Ai), whereas in suckling mice they are diploid (Fig. 3Aii) (34). H19 was significantly decreased in tetraploid mesenchymal cells (283 fold change), as well as in adult mouse livers (4157 fold change), compared to diploid mesenchymal cells and livers of suckling mice,
respectively (Fig. 3B and 3C). As adult livers are comprised of mixed diploid-polyploid cells, the two populations were separated by flow cytometry (Fig. S3A and S3B). It was found that the diploid fraction expressed higher levels of H19 compared to the tetraploid fraction (142 fold change, Fig. 3D). The reduction in H19 expression in the adult mouse was specific to the liver, as no differences were detected in two other epithelial organs (lung and kidney) and neither in the spleen (Fig. 3E). Thus, polyploidy in both normal liver cells and in cultured MSCs is linked with markedly reduced H19 expression compared to diploid cells.

**H19 suppression mediates polyploid phenotypes.** To understand the role of H19, we compared diploid to tetraploid MSCs with respective high and low levels of H19 (Fig. 4Ai), and also performed H19 knock-down in the diploid cells (Fig. 4Aii). The doubling time (Fig. 4Bi and ii), seeding efficiency (4Ci and ii), and UV sensitivity (4Di and ii) of the cells were analyzed. We found that tetraploid cells had a longer population doubling time (Fig. 4Bi) and lower seeding efficiency (Fig. 4Ci) compared to diploid cells. The sensitivity of tetraploid MSCs to UV irradiation was lower than that of diploid cells (Fig. 4Di). Polyploidy was associated with uneven cell divisions and unstable centrosome numbers (17). The stability of the tetraploid MSC genotype in our experiments suggests differently. Examination of centrosome numbers of 2N, 2N+4N, and 4N MSCs showed that most cells contain 1-2 centrosomes (Fig. S4A). Nevertheless, 4N MSCs had a small increase in the proportion of cells with >2 centrosomes (8.2% compared to 4.6-4.8% in 2N and 2N+4N MSCs). Importantly, no multipolar divisions were detected in 4N MSCs (160 mitotic cells inspected), as multiple centrosomes seem to cluster during mitosis (Fig. S4B, C, D), possibly accounting for the stability of the polyploid state (35).

To examine the consequences of reduced H19 expression we specifically knocked it down in diploid MSCs (Fig. 4Aii). Apparently, H19 siRNA caused diploid cells to acquire properties resembling those of tetraploid cells; the doubling time of H19 siRNA treated cells increased (Fig. 4Bii), seeding efficiency decreased (Fig. 4Cii), and UV sensitivity decreased (Fig. 4Dii). Interestingly, H19 expression was elevated, dose dependently, by UV irradiation in tetraploid but not in diploid MSCs, which might explain the relative resistance of tetraploid cells to UV stress (Fig. 4E). However, H19 levels were increased in both diploid (Fig. S5A) and tetraploid (Fig. S5B) cells when reaching confluence. Most importantly, a shift towards polyploidy was detected
after H19 knock-down in diploid cells (Fig. 5A and 5B), which similarly augmented the polyploidization effect of UV irradiation (Fig. 5C and 5D).

We then compared protein expression (Fig S6Ai and ii, and S6Bi and ii), mitotic behavior (S6Ci and ii) and RNA expression (S6Di and ii) in diploid, tetraploid and H19 knocked-down MSCs. Protein analysis revealed that the levels of c-Myc were significantly higher in diploid MSCs (Three-fold difference, Figures S6Ai, Bi). This result is in line with the microarray data (Fig. S2D, E) and with a previous report showing that c-Myc was able to induce H19 expression (36). The levels of p53 as well as AuroraB (regulator of chromosomal segregation) were relatively similar. Also, AuroraB localization was found normal in 20 mitoses of diploid and tetraploid MSCs (Fig. S6Ci). In addition, the gene expression of c-Myc, p21 and p53 was not found to be different between diploid and tetraploid MSCs (Fig. S6Di). H19 knock-down showed no effect on the protein levels of c-Myc and p53 (Figures S6Aii, Bii). However, a possible elevation in the level of AuroraB was detected after H19 knock-down. The significance of this elevation is not clear, since the localization of AuroraB during mitosis after knock-down appeared normal (20 mitoses from each cell were inspected, Fig. S6Cii). The RNA levels of c-Myc, p21 and p53 were not affected by H19 knock-down (Fig. S6Dii). Finally, the levels of c-Myc and p53 proteins, as well as c-Myc, p21 and p53 mRNAs were extremely heterogeneous among the different tumors formed by MSCs (Figures S7A, B). In contrast, H19 expression correlated well with the tumor-forming propensity of the cells.

Tetraploidization of diploid cells reduces tumorigenic potential and lowers H19 expression. In order to critically examine the implied connection between polyploidy associated with decreased H19 expression and tumorigenicity, we tested the effect of artificial tetraploidization (31) on diploid MSCs. Three MSCs (MSC3, 4 and 5) successfully underwent the procedure as evident from the tetraploid G2M cells which formed (Figures 6A and S8A-D). Knock-down of H19 increased the efficiency of artificial tetraploidization, providing further evidence for its role in the control of cell ploidy (Fig. 6B).

Clonal isolates from two of the MSCs driven to tetraploidy showed that the proportion of 4N cells is about ~25% (3/11 and 2/8 4N clones from MSC4 and MSC5 respectively), and that none of these cells had a tumorigenic potential. Artificial tetraploidization of MSC3 (MSC3-tet) also resulted in a significant reduction of the tumorigenicity compared to control treated MSC3.
(MSC3-control, Fig. 6C). The reduction in tumorigenicity of MSC3-tet was accompanied by a reduction in H19 expression (Fig. 6D). The artificial tetraploidization procedure resulted in a population with mixed ploidy, and the diploid cells remained at a significant amount, possibly masking the full extent of H19 reduction in the newly generated tetraploid cells. Therefore, artificial tetraploid clones and their diploid counterparts were isolated from the populations after the artificial tetraploidization procedure. The tetraploid clones were found to express 6.5 fold less H19 than the diploid clones (Fig. 6E). Taken together, these data suggest that artificial tetraploidization of MSCs reduces tumorigenic potential in conjunction with suppression of H19 expression. In contrast, primary human fibroblasts immortalized using hTERT, expressed significantly higher levels of H19 compared to control cells (Fig. 6F). This implies that H19 might also be involved in the transformation of human mesenchyme.
Discussion

Conflicting information related to MSC tumorigenicity has been reported (6-12, 37). Our study shows that tumor formation by mouse MSCs is a relatively rare event. Human cells are by far more stable genetically, and therefore their safety should be even greater (12). Nevertheless, tumorigenic capacity of human cells can be assessed in animal models which provide valuable information but underestimate cell capacities. Therefore, there is a need for alternative ways to assess the tumorigenicity of human cells. In this study, we focused on mouse MSCs to understand what might drive them towards malignant transformation. We found that tumorigenic mouse MSCs highly express H19. Furthermore, normal human fibroblasts transduced by TERT, a process that leads to their transformation, exhibit augmented expression of H19. The analysis of H19 could therefore serve as a marker for the selection of mesenchymal cells which are safe for clinical use. Furthermore, targeting of H19 in mesenchymal tumors, may serve as means to eradicate sarcomas, as already suggested for non-mesenchymal cancers (38).

The inverse relationship between polyploidy and cancer reported here prompted experiments in which polyploidy was forced on diploid cells artificially. As a result, the cells became less cancerous compared with the control diploid population. This intriguing phenomenon substantiates our initial finding, i.e., the higher tumorigenicity of diploid MSCs when compared to that of polyploid ones. It is further implied that enforcement of polyploidy should be considered as means to attenuate tumor progression. Indeed, increased chromosomal content occasionally serves to decrease tumorigenic potential, as in Down’s syndrome (39, 40), or in neuroblastoma (41). Artificial tetraploidization also resulted in the suppression of H19 expression. Similarly, H19 knock-down lead to increased polyploidization. Our study thus establishes a link between H19 expression and cell ploidy.

The majority of mouse MSCs in our study, as well as in other reports, is polyploid (42). In contrast, human MSCs maintain a rather stable chromosome number in culture. This is probably due to their very slow growth rate. Subsequently, the time frame of in vitro studies does not allow for accumulation of chromosomal changes in human MSCs in a way similar to that of mouse cells. From this point of view our data has limited clinical relevance. However, it cannot be excluded, at this point, that the low incidence of polyploidization of human MSCs might predict higher propensity to transformation and cancer. Thus far, MSC transplantation did not
result in their long-term tissue residence neither in mice nor in patients (43). If in the future better engraftment and long-term in vivo survival of human MSCs will be achieved, the possibility that such human diploid cells are potentially dangerous, should be revisited.

It has unequivocally been shown that human cancers are often associated with aneuploidy (14). Chromosomal instability clearly contributes to cancer progression (19) and in this respect the fact that polyploid MSCs are rarely tumorigenic and exhibit properties in common with normal, rather than cancer cells (Fig. 2C and Fig. 4) is surprising. In an attempt to reconcile our findings with the vast literature on the role of CIN in cancer, we propose a modification of the current view: Since culture conditions may impose stress on primary cells it is hypothesized that polyploidization is one possible response, out of many (44-47), to environmental insults. We propose a putative function of polyploidy in counteracting malignancy under environmental stress, such as that imposed on the liver cell population in vivo or on cells in ex vivo culture (Fig. 7). Apparently, cells react to stress by elevating their H19 expression. When stress is excessive (Fig. 7A), diploid transformed cells and chromosomally unstable polyploid cells form, leading to malignancy (48, 49). Alternatively, some cells could avoid malignant transformation by undergoing polyploidization associated with repression of H19 expression (Fig. 7B). Such cells might be resistant to further stress as the numerical changes in chromosomal content possibly buffer structural changes. Indeed, the cytogenetic analysis in Figure 1 supports this option: non-tumorigenic polyploid cells harbor chromosomal abnormalities in single chromosomes, in contrast to tumorigenic cells in which the polyploid sub-population have doubled abnormalities in homologous chromosomes. This means that the perturbation occurred prior to polyploidization. Such events may lead to the emergence of stable polyploid cells that are non-tumorigenic as found in our experiments. The fact that we were able to attenuate the tumorigenicity of diploid cells by in vitro artificial tetraploidization, suggests that doubling the amount of DNA, even at the cost of doubling possible existing genetic abnormalities, still has a positive effect. This might be explained by the associated reduction of H19 in the process. Thus, polyploidization might either have a cancer promoting or a cancer preventing effect, as is the case in aneuploidy (50). Further experiments should determine whether polyploidization occurring in vivo, involves H19 or other unknown molecules, and protects against transformation and subsequent tumorigenesis.
Acknowledgments

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Author Contributions

O.S. and D.Z. designed all of the experiments, analyzed the data and wrote the paper. O.S. performed most of the experiments. H.M., N.S., S.K., S.M. and O.R. participated in specific experimental parts. L.T. performed the cytogenetic work. D.L performed the microarray data analysis D.Z. and G.R. supervised various aspects of this work.
References


Figures

**Figure 1. MSCs acquire polyploidy in culture.** A, SKY of four MSC populations and one MSC clone. B, Possible order of events of the numerical (2N/4N) and structural (marked with *) changes in cultured MSCs (see also supplementary table 2): Tetraploidy with no apparent structural aberrations (MSC20), tetraploidy followed by introduction of several structural aberrations on single homologous chromosomes (such as der(12)t(10;12) in Clone 1), partial tetraploidy followed by introduction of several structural aberrations on single homologous chromosomes (such as der(7)t(7;13) in sub-population B of MSC9), and introduction of structural aberration at the diploid state which multiply upon polyploidization (such as +der(4)t(4;18) in sub-populations A and C doubled in sub-populations B and D of MSC8). C, DNA content of cultured MSCs determined by flow cytometry. The following are MSCs originating from the same derivation: MSC1 (passage 20) and MSC7 (passage 40), MSC2 (passage 10) and MSC8 (passage 20), and MSC3 (passage 20) and MSC9 (passage 10). The ploidy of MSC20 and Clone1 remained constant from passage 10 (marked with *) up to passage 40 (without *). D, Summary of the ploidy of 28 MSC populations and 47 MSC clones (derived from three 2N+4N populations).

**Figure 2. Polyploid MSCs are less tumorigenic than diploid MSCs.** A, Kaplan-Meier plots comparing tumor formation of MSCs with different ploidy originated from the same derivation. Number of mice used and additional information is detailed in Supplementary table 3. B, Representative images of histological sections from tumors formed by MSC1 (fibrosarcoma), and MSC2 and MSC3 (fibro-osteosarcomas). C, The average tumor incidence of each ploidy group (average of tumor incidence of cells in each ploidy group as described in Supplementary tables 4 and 5, average ± s.e.m). * p<0.05 (Kruskal-Wallis test).

**Figure 3. Expression of the non-coding RNA H19 is markedly lower in polyploid cells compared to diploid cells in vitro and in vivo.** A, Ploidy of fresh hepatocytes derived from suckling (i, diploid) and adult (ii, diploid-tetraploid) mice. B, C, D, Comparison of H19 expression (relative to HPRT) in diploid (2N) and tetraploid (4N) cultured mesenchymal cells (B, independent t-test), suckling and adult hepatocytes (C, independent t-test), and purified diploid (2N) and tetraploid (4N) hepatocytes from adult liver (D, linear mixed model). Bars indicate average ± s.e.m. * p<0.0002. E, Levels of H19 expression in suckling and adult non-liver tissues (n=2 in each column). Bars indicate average ± s.d. (n.s. – not significant).

**Figure 4. Suppression of H19 shifts diploid MSCs towards “tetraploid-like” phenotypes.** A, H19 expression: (i) In Two diploid MSCs (3 and 5), and two tetraploid MSCs (25 and 22) (ii) After siRNA knock-down done on MSC5 (representative experiment). Comparison of doubling time (B), seeding efficiency (C), and UV sensitivity (15J/m^2, D) between diploid (2N) and tetraploid (4N) MSCs (i, three experiments, post-hoc Tukey analysis) and between control and H19 siRNA treated diploid MSCs (ii, five experiments, paired t-test). Cells used had at least 70% knock-down. E, H19 expression after UV irradiation in MSC22 (4N) and MSC5 (2N). Experiment was done in triplicates and confirmed using additional three diploid and three tetraploid cells, representative data is shown (student t-test). Bars indicate mean ± s.d. * p<0.0001, ** p<0.005, *** p<0.05.

**Figure 5. Suppression of H19 augments polyploidization of diploid MSCs.** A, H19 knock-down increases the ploidy of MSC5 as seen from the decrease in 2N(G1), increase in 2N(G2M)+4N(G1), and increase in the polyploid fraction (poly) (Five experiments, paired t-test). B, One representative experiment out of five shown in panel A. C, H19 knock-down increases the ploidy of MSC5 after 15J/m^2 UV irradiation. D, One representative experiment out of four shown in panel C. Bars indicate average ± s.d. Analysis done using paired t-test. * p<0.0055.

**Figure 6. Tetraploidization reduces tumorigenicity and lowers H19 expression in mouse MSCs, and immortalization increases H19 expression in human primary fibroblasts.** A, Artificial tetraploidization of MSC3 yields a 4N sub-population (MSC3-tetra) observed in flow cytometry. Control treated cells remain diploid (MSC3-control). B, H19 knock-down increases the efficiency of artificial tetraploidization of MSC3 (representative of three experiments is shown). C, Kaplan-Meier plot of MSC3-control and MSC3-tetra after subcutaneous injection into immune-deficient mice (10^5 cells). D, Reduction
of H19 levels in MSC3-tetra compared to MSC3-control. E Reduction of H19 levels in tetraploid clones compared to diploid clones derived from MSC populations after artificial tetraploidization. Bars indicate average ± s.d. Analysis done using independent t-test. * p<0.015. F, Comparison of H19 expression levels between normal and immortalized (TERT) primary human fibroblasts. Bars indicate average ± s.e.m. ** p=0.014 (paired t-test).

**Figure 7. Some possible cell fates under stress.** Cells under stress with increased H19 levels can undergo transformation and become cancerous cells, also through CIN (A, red background). This study implies that alternatively, tetraploidy accompanied by H19 suppression can maintain a non-cancerous state (B, green background). Tetraploidization of cancerous diploid cells can attenuate their tumorigenic properties (“Evasion”) and reduce H19 expression. The in vivo occurrence of such an event remains to be examined.
Figure 3 Shoshani et al.

### A

- **Suckling liver**
  - 2N: 80%

- **Adult liver**
  - 2N: 8.26%
  - 4N: 62.3%

### B

**Relative H13 expression**

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>2N (n=4)</th>
<th>4N (n=6)</th>
<th>2N (n=3)</th>
<th>4N (n=7)</th>
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<td>Mesenchymal cells</td>
<td><img src="image" alt="Graph" /></td>
<td><img src="image" alt="Graph" /></td>
<td><img src="image" alt="Graph" /></td>
<td><img src="image" alt="Graph" /></td>
</tr>
<tr>
<td>Suckling liver</td>
<td><img src="image" alt="Graph" /></td>
<td><img src="image" alt="Graph" /></td>
<td><img src="image" alt="Graph" /></td>
<td><img src="image" alt="Graph" /></td>
</tr>
<tr>
<td>Adult liver</td>
<td><img src="image" alt="Graph" /></td>
<td><img src="image" alt="Graph" /></td>
<td><img src="image" alt="Graph" /></td>
<td><img src="image" alt="Graph" /></td>
</tr>
<tr>
<td>Adult sorted liver</td>
<td><img src="image" alt="Graph" /></td>
<td><img src="image" alt="Graph" /></td>
<td><img src="image" alt="Graph" /></td>
<td><img src="image" alt="Graph" /></td>
</tr>
</tbody>
</table>

### C

- **Relative H13 expression**

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>2N (n=4)</th>
<th>4N (n=6)</th>
<th>2N (n=3)</th>
<th>4N (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mesenchymal cells</td>
<td><img src="image" alt="Graph" /></td>
<td><img src="image" alt="Graph" /></td>
<td><img src="image" alt="Graph" /></td>
<td><img src="image" alt="Graph" /></td>
</tr>
<tr>
<td>Suckling liver</td>
<td><img src="image" alt="Graph" /></td>
<td><img src="image" alt="Graph" /></td>
<td><img src="image" alt="Graph" /></td>
<td><img src="image" alt="Graph" /></td>
</tr>
<tr>
<td>Adult liver</td>
<td><img src="image" alt="Graph" /></td>
<td><img src="image" alt="Graph" /></td>
<td><img src="image" alt="Graph" /></td>
<td><img src="image" alt="Graph" /></td>
</tr>
<tr>
<td>Adult sorted liver</td>
<td><img src="image" alt="Graph" /></td>
<td><img src="image" alt="Graph" /></td>
<td><img src="image" alt="Graph" /></td>
<td><img src="image" alt="Graph" /></td>
</tr>
</tbody>
</table>

### D

- **Relative H13 expression**

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>2N (n=4)</th>
<th>4N (n=6)</th>
<th>2N (n=3)</th>
<th>4N (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mesenchymal cells</td>
<td><img src="image" alt="Graph" /></td>
<td><img src="image" alt="Graph" /></td>
<td><img src="image" alt="Graph" /></td>
<td><img src="image" alt="Graph" /></td>
</tr>
<tr>
<td>Suckling liver</td>
<td><img src="image" alt="Graph" /></td>
<td><img src="image" alt="Graph" /></td>
<td><img src="image" alt="Graph" /></td>
<td><img src="image" alt="Graph" /></td>
</tr>
<tr>
<td>Adult liver</td>
<td><img src="image" alt="Graph" /></td>
<td><img src="image" alt="Graph" /></td>
<td><img src="image" alt="Graph" /></td>
<td><img src="image" alt="Graph" /></td>
</tr>
<tr>
<td>Adult sorted liver</td>
<td><img src="image" alt="Graph" /></td>
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</tbody>
</table>

### E

**Relative H13 expression**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Suckling</th>
<th>Adult</th>
<th>Suckling</th>
<th>Adult</th>
<th>Suckling</th>
<th>Adult</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spleen</td>
<td><img src="image" alt="Graph" /></td>
<td><img src="image" alt="Graph" /></td>
<td><img src="image" alt="Graph" /></td>
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<tr>
<td>Lung</td>
<td><img src="image" alt="Graph" /></td>
<td><img src="image" alt="Graph" /></td>
<td><img src="image" alt="Graph" /></td>
<td><img src="image" alt="Graph" /></td>
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<tr>
<td>Kidney</td>
<td><img src="image" alt="Graph" /></td>
<td><img src="image" alt="Graph" /></td>
<td><img src="image" alt="Graph" /></td>
<td><img src="image" alt="Graph" /></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* n.s. = not significant
Figure 4 Shoshani et al.

A. Relative H19 expression

B. Doubling time

C. Seeding efficiency

D. UV sensitivity

E. Relative H19 expression

- (A) i: MSC5, MSC3, MSC25, MSC22 (2N), (H19 KD) 0.0001 - 10
- (A) ii: Control, H19 KD

- (B) i: MSC5, MSC3, MSC25, MSC22 (2N), (H19 KD) 0.000001 - 0.001
- (B) ii: Control, H19 KD

- (C) i: MSC5, MSC3, MSC25, MSC22 (2N), (H19 KD) 0 - 30
- (C) ii: Control, H19 KD

- (D) i: MSC5, MSC3, MSC25, MSC22 (2N), (H19 KD) 0 - 120
- (D) ii: Control, H19 KD

- (E) Relative H19 expression

- UV intensity

- p = 0.0145

- 0J, 5J, 10J, 15J
Figure 5 Shoshani et al.

A

B

C

D

Control | H19 KD | Control | H19 KD | Control | H19 KD

2N (G1) | p=0.0142

2N (G2M) + 4N (G1) | p=0.0197

p=0.0023

Control | H19 KD

2N (G1) 49.3%

2N (G2M) + 4N (G1) 33.4%

poly 6.09%

FL2-A

Control | H19 KD

2N (G1) 42.9%

2N (G2M) + 4N (G1) 33.8%

poly 10.4%

FL2-A

Control | H19 KD

2N (G1) 41.1%

2N (G2M) + 4N (G1) 38.2%

poly 10.7%

FL2-A

Control | H19 KD

2N (G1) 33.5%

2N (G2M) + 4N (G1) 41.2%

poly 15.5%

FL2-A
Figure 7 Shoshani et al.
Polyploidization of murine mesenchymal cells is associated with suppression of the long non-coding RNA H19 and reduced tumorigenicity

Ofer Shoshani, Hassan Massalha, Nir Shani, et al.

Cancer Res  Published OnlineFirst October 9, 2012.

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