Circulation

RESEARCH LETTER

Telomeres Fuse During Cardiomyocyte Maturation

Esther Aix, PhD*; Alex Gallinat, PhD*; Carla Yago-Díez, PhD; Javier Lucas, MS; Manuel José Gómez, PhD; Alberto Benguría, PhD; Patricia Freitag; Elizabeth Cortez-Toledo; Laura Fernández de Manuel, PhD; Lucía García-Cuasimodo, BS; Héctor Sánchez-Iranzo, PhD; María C. Montoya, PhD; Ana Dopazo, PhD; Fátima Sánchez-Cabo, PhD; Nadia Mercader, PhD; Javier E. López, MD; Bernd K. Fleischmann, MD; Michael Hesse, PhD; Ignacio Flores, PhD

Although polyploidization is a hallmark of adult mammalian cardiomyocytes and may constrain their proliferation, the mechanisms leading to ploidy increase in cardiomyocytes remain elusive.[1] Our laboratory and others have reported the formation of DNA bridges between daughter nuclei as a potential route to cardiomyocyte polyploidization.[2,3] These earlier in vivo studies found DNA bridges on thin tissue sections, but this approach does not cover all events because not all cardiomyocytes were oriented in the same 2-dimensional plane.

To detect all DNA bridges, we dispersed cardiomyocytes obtained from Myh6-H2B-mCh mice[4] at postnatal day 4 on a culture plate and labeled them with the mitosis marker phospho-histone H3 (pH3) and DAPI (4'6-diamidino-2-phenylindole). No apparent mitotic errors were identified in prophase or metaphase, but DNA bridges were apparent in 54±7.9% of cardiomyocytes in early anaphase (Figure [A and B]). The percentage of cardiomyocytes with DNA bridges decreased rapidly to 17.8±9.5% in late anaphase. On the contrary, the percentage of cardiomyocytes with resolved or broken DNA bridges (cardiomyocytes with discontinuous DNA fibers between sister chromatids) increased from 11.7±6.4% in early anaphase to 28.9±16.3% in late anaphase. DNA bridge remnants were present in 8±3.7% of telophase cardiomyocytes. This analysis indicates that DNA bridges form in early anaphase and are resolved in the subsequent mitotic stages. Time-lapse videomicroscopy of postnatal day 4 cardiomyocytes reinforces the finding that DNA bridges between sister chromatids resolve as mitosis advances (Figure [C]).

DNA bridges can form as the result of diverse physical connections. Of these connections, covalently ligated telomere fusions can arguably operate in postnatal cardiomyocytes because after birth, telomerase activity declines and cardiomyocyte telomeres shorten very rapidly and become dysfunctional, being susceptible to nonhomologous end joining.[2] To analyze whether telomeres fuse in mouse hearts after birth, we sequenced telomere-enriched genomic DNA obtained from postnatal day 1 and postnatal day 8 hearts to search for reads containing at least three 5'-TTAGGG telomere sequences followed by 3 or more CCCTAA-3' reverse-complementary telomere sequences[5] (Figure [D]). Analysis of telomere-enriched sequences identified a 1.8-fold increase of telomere fusions in cardiac cells and a 1.6-fold increase in purified ventricular cardiomyocytes during the process of DNA bridging and binucleation (Figure [E]). The hypothesis that telomeres fuse to generate polyploid cardiomyocytes predicts that telomere fusion will be less frequent in diploid than in polyploid cardiomyocytes. Comparison of the mononuclear and binuclear fractions of sorted postnatal day 7 cardiomyocytes revealed a 227% increase in the number of telomere fusions in binuclear cardiomyocytes (Figure [F]). These findings indicate that telomere fusions are present in newborn cardiomyocytes and increase in frequency during DNA bridging and binucleation.

To assess whether telomere fusions occur in other species relevant to the cardiovascular research, we repeated the telomere enrichment and sequence analysis on DNA extracted from zebrafish, pig, and human hearts (Figure [G]). We found reads containing telomere fusion sequences in all these species. Frequencies of

Key Words: myocytes, cardiac ■ polyploidy ■ telomere

Correspondence to: Ignacio Flores, PhD, Nicolas Cabrera 1, Campus UAM, E-28049 Madrid, Spain. Email iflores@cnic.es or iflores@cbm.csic.es
*E. Aix and A. Gallinat contributed equally.
For Sources of Funding and Disclosures, see page 1636.

Circulation is available at www.ahajournals.org/journal/circ

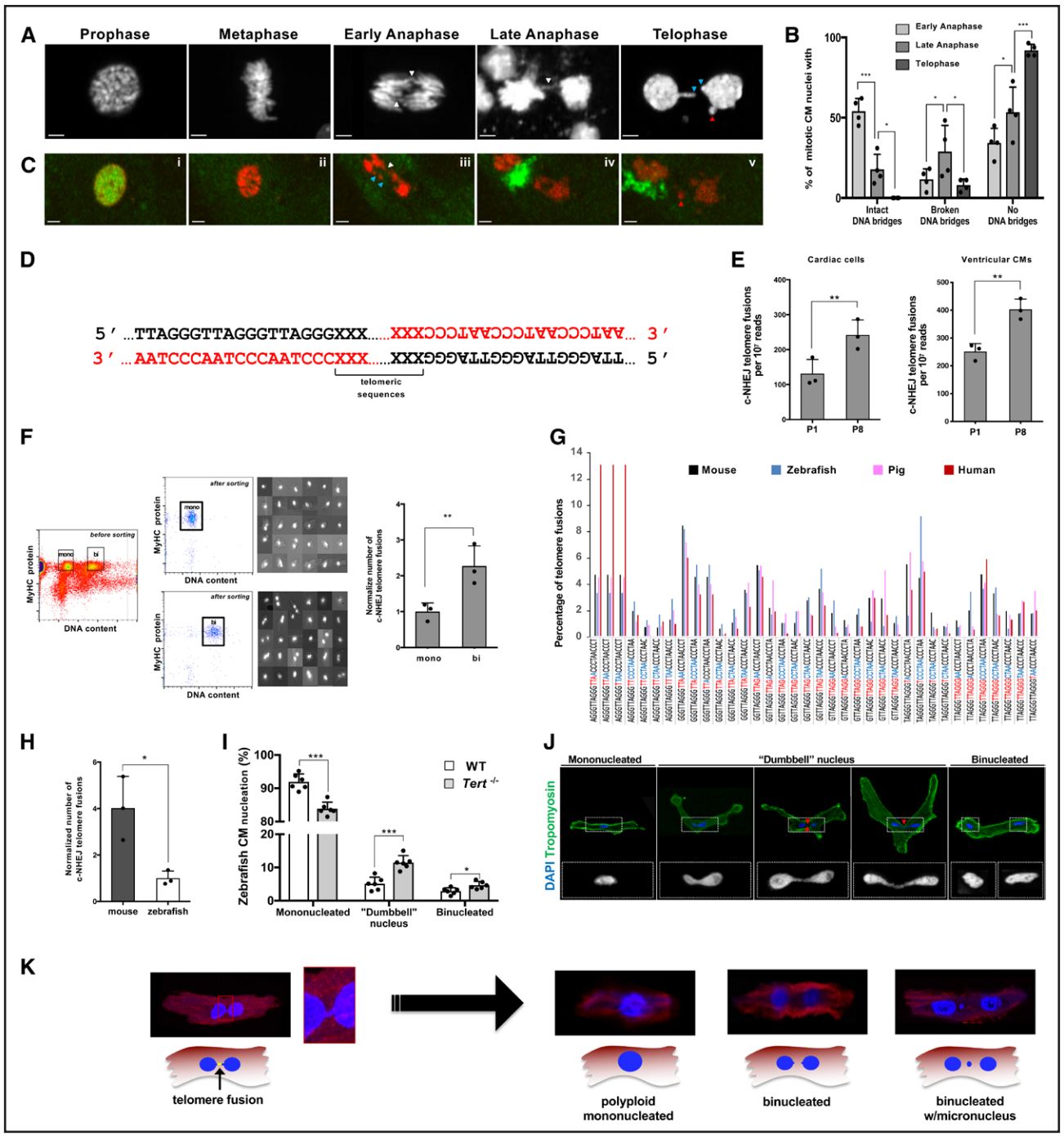

Figure. Telomeres fuse during cardiomyocyte maturation.

A, Chromosome bridging and subsequent resolution in postnatal mouse cardiomyocytes. Examples of nuclear division abnormalities appearing in postnatal day 4 pH3^{+}-cardiomyocytes from early anaphase: DNA bridges (white arrowheads), resolved/broken DNA bridges (blue arrowheads), and micronucleus (red arrowhead; bars=4 μM). **B**, Percentage of mitotic (pH3^{+}) cardiomyocytes with intact DNA bridges, broken DNA bridges, and no DNA bridges in early anaphase, late anaphase, and telophase. A total of ≈150 000 cardiac cells from 4 postnatal day 4 mouse hearts were examined by automated high-content image screening. Between-group differences were analyzed by 2-way ANOVA followed by Tukey-Kramer post hoc comparisons (*P<0.05; ***P<0.001). **C**, Time-lapse imaging of eGFP-anillin (green) and H2B-mCh (red) in a cardiomyocyte during mitosis. An intact DNA bridge (white arrowhead) and a broken DNA bridge (blue arrowheads) are observed in frame iii, and a micronucleus in frame v. Anillin, a multidomain protein involved in the closure of the contractile ring during cytokinesis, converged at the described midzone during late anaphase but was displaced laterally at the end of mitosis (frames iv–v; bars=4 μM). **D**, Telomere fusions generated by classic nonhomologous end-joining (c-NHEJ). **E**, Postnatal increases in telomere fusions in cardiac cells (**left**) and purified cardiomyocyte (**right**). Data are shown as the mean±SD of 3 independent experiments (**P<0.01; Student t test). **F**, Binucleated cardiomyocytes contain more telomere fusions than mononucleated cardiomyocytes. FACS enrichment of P7 mononucleated vs binucleated cardiomyocytes before sorting (**left**) and after sorting (**middle**). **Right**, Normalized number of telomere fusions in mononucleated and binucleated cardiomyocytes. (*Continued*)

Figure Continued. Data are shown as the mean±SD of 3 independent experiments (**P<0.01; Student t test). **G**, Telomere fusions follow a conserved and distinctive pattern. Frequency of sequences at the telomere fusion junction in mouse, zebrafish, pig, and human hearts. Red and blue letters highlight the telomeric sequences at the fusion point. **H**, Telomere fusion frequency correlates with the percentage of binucleated cardiomyocytes in mouse and zebrafish hearts. Normalized number of telomere fusions in cardiac genomes from 3-month-old mouse and zebrafish hearts. Data are shown as the mean±SD of 3 independent experiments (*P<0.05; Student t test). **I**, Percentage of mononucleated, dumbbell, and binucleated cardiomyocytes in wild-type and Tert$^{-/-}$ zebrafish cardiomyocytes. The circularity of dumbbell cardiomyocyte nuclei is <0.4. Data are shown as the mean±SD of 6 independent experiments (*P<0.05; ***P<0.001; Student t test). **J**, Examples of mononucleated (**left**), dumbbell (**middle**), and binucleated cardiomyocytes (**right**) from zebrafish hearts. **K**, DNA bridging and rupture as the origin of polypoid cardiomyocytes and cardiomyocyte proliferation blockade. Formation of DNA bridges as a result of telomere fusion might explain the genesis of the main types of polyploid cardiomyocytes seen in the mature heart. A tetraploid cardiomyocyte with one nucleus would form if the DNA bridge or bridges persist and the 2 sister chromatids rejoin. A binucleated cardiomyocyte would form if the DNA bridge or bridges break at one site. A binucleated cardiomyocyte containing a fragmented DNA micronucleus would form if the DNA bridge or bridges break at 2 or more sites. An origin in telomere fusion might also explain why polyploid cardiomyocytes do not usually divide. Tetraploidy and chromosome breakage are early and late consequences of telomere fusions, respectively, and both are associated with a DNA damage response (DDR) that ultimately prevents cell division. MyHC indicates myosin heavy chain.

permutations of the TTAGGG-CCCTAA sequence were similar at the junction in mouse, zebrafish, pig, and human hearts, indicating a conserved need for specific bases at the breakpoint across these species.

Unlike mice, pigs, and humans, zebrafish maintain the capability for heart regeneration and cardiomyocyte proliferation well beyond the neonatal period. The proliferation competence of adult zebrafish cardiomyocytes correlates with their mostly diploid nuclear DNA content.[1] To determine whether ploidy in mouse and zebrafish hearts correlates with the abundance of telomere fusions, we extracted DNA from 3-month-old mouse hearts and 3-month-old zebrafish hearts. Analysis after the enrichment and sequencing step detected an average of 4.0 fewer telomere fusions in the adult zebrafish heart than in the adult mouse heart (Figure [H]). To determine whether generation of binucleated cardiomyocytes in zebrafish is influenced by telomerase, we isolated cardiomyocytes from wild-type and telomerase-null 3-month-old zebrafish. We detected a small fraction of binucleated cardiomyocytes in wild-type zebrafish hearts, as previously reported[1] (Figure [I and J]). We also detected a distinct cardiomyocyte subpopulation containing one elongated nucleus with a circularity factor <0.4, suggesting the presence of DNA bridges between 2 separating DNA masses. The proportion of binucleated and dumbbell-shaped cardiomyocytes was higher in the hearts of telomerase-null zebrafish. Cytoplasm division in dumbbell cardiomyocytes seems to stop precisely at the location of the bridge, suggesting that DNA bridging physically obstructs cytokinesis (Figure [J]).

Our findings uncovered telomere fusions as a potential molecular mechanism underlying the formation of DNA bridges in polyploid cardiomyocytes, which might explain the genesis of the main types of polyploid cardiomyocytes seen in the mature heart and why polyploid cardiomyocytes do not usually divide (Figure [K]). Future research will be needed to determine whether telomere fusion plays a direct role in ploidy increases during development, growth, and aging, and whether it can be manipulated to enhance the plasticity of the mammalian myocardium in the event of injury.

All animal procedures complied with the Institutional Animal Care and Use Committee. The institutional review board has approved procedures and documents to obtain informed consent. The data that support the findings of this study and study materials, as well as experimental procedures and protocols, are available from the corresponding author on reasonable request.

ARTICLE INFORMATION

Affiliations

Centro Nacional de Investigaciones Cardiovasculares Carlos III (CNIC), Madrid, Spain (E.A., A.G., C.Y.-D., J.L., M.J.G., A.B., L.F.d.M., L.G.-C., H.S.-I., M.C.M., A.D., F.S.-C., N.M., I.F.). Institute of Physiology I, Life and Brain Center, University of Bonn, Germany (P.F., B.K.F., M.H.). University of California, Davis (E.C.-T., J.E.L.). IBCS-BIP, Karlsruhe Institute of Technology, Eggenstein-Leopoldshafen, Germany (H.S.-I.). Institute of Anatomy, University of Bern, Switzerland (N.M.). Centro de Biologia Molecular Severo Ochoa, CSIC-UAM, Cantoblanco, Madrid, Spain (I.F.).

Acknowledgments

The authors thank the Animal Facility and Microscopy Unit from Centro Nacional de Investigaciones Cardiovasculares Carlos III and M. Galardi for zebrafish husbandry.

Sources of Funding

Dr Mercader has been funded by SNF grant 320030E-164245 and ERC Consolidator grant 2018 819717. Drs Fleischmann and Hesse have been funded by the German Research Foundation (FOR1352 and FOR2743). Dr Flores was funded by grants from the Spanish Ministry of Science and Innovation (PID2019-110339RB-I00) and the Comunidad de Madrid (S2017/BMD-3875 and S2022/BMD-7245). The CNIC and CBM-SO are supported by the Ministerio de Ciencia e Innovación and are Severo Ochoa Centers of Excellence (CEX2020-001041-S, CEX2021-001154-S).

Disclosures

None.

REFERENCES

1. Kirillova A, Han L, Liu H, Kühn B. Polyploid cardiomyocytes: implications for heart regeneration. *Development*. 2021;148:dev199401. doi: 10.1242/dev.199401
2. Aix E, Gutierrez-Gutierrez O, Sanchez-Ferrer C, Aguado T, Flores I. Postnatal telomere dysfunction induces cardiomyocyte cell-cycle arrest through p21 activation. *J Cell Biol*. 2016;213:571–583. doi: 10.1083/jcb.201510091
3. Engel FB, Schebesta M, Keating MT. Anillin localization defect in cardiomyocyte binucleation. *J Mol Cell Cardiol*. 2006;41:601–612. doi: 10.1016/j.yjmcc.2006.06.012
4. Raulf A, Horder H, Tarnawski L, Geisen C, Ottersbach A, Roll W, Jovinge S, Fleischmann BK, Hesse M. Transgenic systems for unequivocal identification of cardiac myocyte nuclei and analysis of cardiomyocyte cell cycle status. *Basic Res Cardiol*. 2015;110:1–18. doi: 10.1007/s00395-015-0489-2
5. Mateos-Gomez PA, Gong F, Nair N, Miller KM, Lazzerini-Denchi E, Sfeir A. Mammalian polymerase theta promotes alternative NHEJ and suppresses recombination. *Nature*. 2015;518:254–257. doi: 10.1038/nature14157