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Chondrogenic Differentiation of Amniotic Fluid Stem Cells and Their Potential for Regenerative Therapy

Andrea Preitschopf · Hannes Zwickl · Kongzhao Li · Gert Lubec · Gabor Joo · Margit Rosner · Markus Hengstschläger · Mario Mikula

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Abstract Chronic articular cartilage defects are the most common disabling conditions of humans in the western world. The incidence for cartilage defects is increasing with age and the most prominent risk factors are overweight and sports associated overloading. Damage of articular cartilage frequently leads to osteoarthritis due to the aneural and avascular nature of articular cartilage, which impairs regeneration and repair. Hence, patients affected by cartilage defects will benefit from a cell-based transplantation strategy. Autologous chondrocytes, mesenchymal stem cells and embryonic stem cells are suitable donor cells for regeneration approaches and most recently the discovery of amniotic fluid stem cells has opened a plethora of new therapeutic options. It is the aim of this review to summarize recent advances in the use of amniotic fluid stem cells as novel cell sources for the treatment of articular cartilage defects. Molecular aspects of articular cartilage formation as well as degeneration are summarized and the role of growth factor triggered signaling pathways, scaffolds, hypoxia and autophagy during the process of chondrogenic differentiation are discussed.

Keywords Amniotic fluid stem cells · Chondrogenic differentiation · Cartilage · Stem cell based therapy · Osteoarthritis

A. Preitschopf · K. Li · M. Rosner · M. Hengstschläger · M. Mikula (✉)
Institute of Medical Genetics, Medical University of Vienna,
Währinger Strasse 10,
1090 Vienna, Austria
e-mail: mario.mikula@meduniwien.ac.at

H. Zwickl
Center for Regenerative Medicine, Danube University Krems,
Krems, Austria

K. Li · G. Lubec
Department of Pediatrics and Adolescent Medicine,
Medical University of Vienna,
Währinger Gürtel 18,
1090 Vienna, Austria

G. Joo
1st Department of Obstetrics and Gynaecology,
Semmelweis University Medical School,
Baross utca 27,
Budapest 1088, Hungary

Introduction

The ability for physical movement depends on the correct function of articular joints. Hence extensive research activities focus on the developmental programs activated during joint formation and on the mechanisms of articular cartilage homeostasis. Articular cartilage starts to form during fetal joint development and subsequently covers the surface at the bony ends of articular joints, where it is maintained throughout life. The most common form of articular cartilage damage is osteoarthritis (OA), which is also described as a primarily non-inflammatory, degenerative joint disease. Its main histologic features include disruption of articular cartilage, degradation of extracellular matrix, and reduced cellularity within cartilage [1]. The incidence of OA is still increasing and it is the single most common cause of disability in older adults [2]. Cartilage is devoid of nerves and vasculature and hence repair after injury is limited due to poor recruitment of extrinsic cells. Today autologous chondrocyte implantation (ACI) is the treatment of choice for severe cartilage defects [3–5], but this procedure suffers from several drawbacks. These include that the patient has to undergo multiple surgeries, once to isolate chondrocytes by a biopsy from less weight bearing areas of the joint and a second time for re-transplantation of in vitro expanded cells. In addition, in vitro expansion of chondrocytes is slow and

dedifferentiation processes occur, which reduce the ability of cells to produce extracellular matrix [6, 7]. Stem cells isolated from various adult tissues have been tested for their use as cell sources to regenerate chondrogenic tissue [8]. Transplantation of differentiated mesenchymal stem cells remains a promising approach for regenerative therapies, but often the tissue generated in vivo shows lower matrix accumulation and subsequent less mechanical stability as compared to chondrocyte-based transplantations [9, 10]. Amniotic fluid stem (AFS) cells provide hallmarks, which make them superior candidates for cell based therapy approaches and in particular for the treatment of cartilage defects. AFS cells can be biobanked, are genetically stable, immunoprivileged, non tumorigenic and there are no ethical concerns involved in their usage, which is the case for embryonic stem cells [11–15]. Here we review the current knowledge on the differentiation of AFS cells towards chondrocytes. We specially focus on signaling pathways and scaffolds instrumental for differentiation to articular cartilage chondrocytes and we also review signaling pathways able to prevent onset of hypertrophy. The incidence of degenerative joint diseases increases with age and recently the process of autophagy has been identified to counteract age related changes. Therefore we also discuss the role of autophagy for generation and maintenance of mature chondrocytes.

Articular Cartilage Formation and Organization

The only cell type within healthy cartilage is the chondrocyte and it is responsible for the synthesis and maintenance of the cartilaginous matrix. During embryonic development chondrocytes develop from mesenchymal progenitors and they are responsible for endochondral ossification as well as for populating the outer area of articular joints. Chondrocytes constituting articular cartilage show some functional and structural differences from growth plate chondrocytes. Whereas the latter undergo highly dynamic changes as long bone growth persists, articular cartilage chondocytes provide continuous function throughout life. There is evidence that articular chondrocytes originate from a pool of growth differentiation factor 5 (GDF5) positive progenitor cells and that growth plate chondrocytes are derived from GDF5 negative cell populations [16]. GDF5 expression is first evident in the mesenchymal interzone of early joints [17, 18]. The interzone is an essential regulator region and it has been widely assumed that it is responsible for formation of joint structures including articular cartilage, ligaments and synovial lining [19]. Molecular studies have revealed that interzone cells express a number of genes able to control joint development, like: GDF5, WNT5A, WNT4, GLI3, CD44, ERG and NOGGIN [20–23]. Interestingly, GDF5 has chondrogenic activity, whereas WNT ligands and NOGGIN are anti-chondrogenic [20, 23]. Accordingly spatio-temporal signaling starting from the interzone requires pro- and anti-chondrogenic activities for the formation of functional joints. Subsequently during embryonic development the cartilage anlagen separate and form a functioning synovial cavity. This event is accompanied by the loss of collagen type I and expression of collagen type II [24]. One key regulatory molecule in this process is the transcription factor SOX9. It determines mesenchymal progenitor cell differentiation and its molecular function includes binding to the promoter region of collagen type 2, 9, 11, aggrecan, cartilage link protein and cartilage oligomeric matrix protein [25–29]. SOX9 acts in concert with SOX5 and SOX6, the so called SOX-trio genes. The expression of these genes is linked to induction and maintenance of a chondrogenic phenotype [30]. Joint cavitation further involves differential hyaluronan synthesis under the influence of mechanical stimuli. The hyaluronan receptor, CD44, is expressed at the joint interzone and at developing articular surfaces and it induces cell adhesion as well as cell separation, depending on the concentration of hyaluronan [24, 31]. Once the articular joint is established, articular chondrocytes ensure production of extracellular matrix (ECM) components like aggrecan, collagen II and sulfated glycosaminoglycans (sGAG) and consequently provide function throughout life. Mature articular cartilage consists of three zones: the superficial zone, where small and flat chondrocytes reside and collagen fibrils are orientated parallel to the surface, the transitional zone, representing the thickest part of cartilage, where collagen fibers are less organized, and the radial zone, where fibers are orientated perpendicular to the surface and oxygen tension is below 1 % [32]. Underneath is the tidemark, which delineates hyaline articular cartilage from calcified cartilage.

Articular Cartilage Degeneration

Persistent breakdown of joint cartilage frequently leads to OA. Primary OA develops without a known cause of joint degeneration and genetic, biomechanical or biochemical factors often play a role [33, 34]. In many cases articular cartilage injuries are caused by trauma or less commonly by developmental, metabolic or neurologic disorders, this group of conditions is referred to as secondary OA [35]. The majority of individuals over the age of 65 show evidence for OA and hands, knees, hips and spine display symptoms of inflammation, pain and loss of mobility [36]. All joint structures are affected by OA, but articular cartilage is primarily subjected to degeneration. During the initial phase of the disease chondrocytes undergo clustering and show increased cell proliferation and general up-regulation of specific extra cellular matrix components, probably

because residing chondrocytes attempt to compensate for structural changes. If the defects persist, proteinases like matrix metalloproteinases (MMPs) and aggrecanases are expressed and type II collagen and proteoglycans are degraded [37]. The excessive reorganization of hyaline cartilage finally leads to fibrillation of the surface and localized production of fibrocartilage. During progressive OA thickening of the zone of calcified cartilage was monitored [38]. This increase in bone mass could be due to induction of hypertrophy in articular chondrocytes, which results in the release of pro-angiogenic factors and the subsequent apoptosis of chondrocytes [39]. Hypertrophy in articular chondrocytes is reminiscent of hypertrophy during endochondral ossification, where chondrocytes undergo several developmental stages, starting from: precartilage condensation, early differentiation, full differentiation and finally post-mitotic hypertrophy [40]. Upon hypertrophy, growth plate chondrocytes downregulate SOX9 and type II collagen expression, whereas RUNX2, type X collagen, alkaline phosphatase and VEGFA are upregulated [41–43]. Subsequently growth plate chondrocytes undergo apoptosis and the tissue becomes calcified. The process of hypertrophy is therefore undesirable in the articular joint because it leads to a reduction in the number of functional chondrocytes and it can also lead to focal calcification of joint cartilage.

AFS Cells as A Novel Source for the Generation of Articular Chondrocytes

Current treatment options of OA, which remains symptomatic in spite of medical and physiotherapeutic treatment, include reconstructive surgical approaches. For focal cartilage defects marrow–stimulating procedures are often applied [35]. During this approach the subchondral cavity is perforated resulting in blood clot formation and subsequent generation of fibrocartilage. Fibrocartilage is structurally and biomechanically inferior to articular cartilage, still marrow-stimulating procedures results in a temporary relieve of OA associated pain. ACI is another method where chondrocytes are isolated from less weight bearing areas of the joint, expanded in vitro and retransplanted into the defective area. This method suffers from increased donor site morbidity, loss of chondrocyte function after in vitro expansion and also fibrocartilage formation at the site of the defect [6, 7]. Recently, the use of stem cells boosted therapeutic options available in regenerative therapy. Embryonic stem cells are pluripotent, but their use raises ethical concerns and additionally upon transplantation, they potentially give rise to tumors [44, 45]. Also mesenchymal stem cells are available for chondrogenic differentiation and they can be isolated from various adult tissues including the bone marrow, muscle and adipose tissue [8, 46, 47]. These cells harbor limited proliferative capacity and, when isolated from older patients, a mutation free status cannot be guaranteed. The identification of AFS cells enabled novel approaches in regenerative medicine [48–50]. AFS cells can be isolated from amniotic fluid by immune-selecting cells positive for c-Kit. They can be expanded in cell culture and show expression of CD29, CD44, CD73, CD90, CD105 and SSEA4 with over 90 % of cells being positive for OCT4 [11]. They can be grown in large quantities and show a higher differentiation potential compared to adult stem cells [51]. AFS cells harbor a very low risk for tumor development and do not raise the ethical issues of embryonic stem cells. Compared to induced pluripotent stem cells, AFS cells do not need ectopic factors to induce pluripotency, are chromosomally stable and do not harbor the epigenetic memory and accumulated somatic mutations frequently found in adult source cells [11, 51–53]. Accordingly, in the recent years, AFS became increasingly accepted as an optimal tool for basic research and probably also for specific cell-based therapies [11, 54–57]. First studies on the chondrogenic potential of mesenchymal amniocytes were conducted in ovines [58]. Cytokeratin 8, Cytokeratin 18 and Vimentin positive amniotic fluid cells were isolated and grown in chondrogenic medium containing TGF-β2 and IGF-I on polyglycolic acid scaffolds for up to 12 weeks. Compared to fetal hyaline cartilage the engineered constructs displayed less amounts of type II collagen, but similar amounts of glycosaminoglycans. First reports on human AFS cells used either fibroblastoid-type cells obtained from amniotic fluid at the time of birth or c-kit magnetically sorted cell populations, which were capable of increased production of type II collagen and sGAG after treatment with TGF-β1 and IGF-I [59, 60]. Comparing c-kit positive and negative populations for their chondrogenic potential revealed that the c-kit positive stem cell population, after differentiation, displayed significantly higher alcian blue staining and therefore stronger chondrogenic differentiation [61]. Another study, which did not employ c-kit sorted amniotic fluid cells, showed that both amniotic fluid and mesenchymal stem cells could be differentiated to chondrocytes in vitro and in vivo at comparable rates [62]. These findings emphasize that supplementation of chondrogenesis promoting factors is necessary for successful manipulation of AFS cells and that the isolation of c-kit positive cells also enhances the differentiation process.

Growth Factors Regulate Chondrocyte Differentiation

Further elucidating the molecular regulation of articular joint formation during development will aid in creating superior working protocols for the differentiation of articular chondrocytes from stem cells. What is known until now is

that the formation of the interzone as well as the subsequent cavitation process and morphogenesis is subjected to strict temporal and spatial regulations and it has become clear that pro- as well as anti-chondrogenic stimuli are needed for successful joint formation [40]. GDF5 was the first marker to be identified in the developing joint and it has been shown to promote initiation of chondrogenesis and also proliferation of chondrocytes [17]. GDF5 belongs to the transforming growth factor β (TGF-β) superfamily of cytokines, which also includes bone morphogenic proteins (BMP). Generally, TGF-β family members stimulate chondrogenesis and for example GDF5 over-expression results in cartilage overgrowth and joint fusion in vivo [63, 64]. Therefore TGF-β family members are frequently used, most often in combination with insulin like growth factor I (IGF-I), to initiate differentiation of AFS cells and for example TGF-β3 stimulation has been shown to be sufficient for chondrogenesis in amniotic fluid derived cells as well as in bone marrow derived mesenchymal stem cells [62]. But recent data has also shown that in vitro expanded autologous chondrocytes, when treated with TGF-β1, show onset of hypertrophy, an undesirable effect for cells possibly intended for tissue-engineered cartilage repair [65].

During progression of development at the interzone also anti-chondrogenic factors are needed to control coordinate formation of joint structures. Noggin is a bone morphogenic antagonist identified to play an essential role during joint formation, since its deletion results in hyperplasia and affected mice do not form joints [20]. WNT-4, WNT-14, WNT-16 and the WNT signaling mediator β-catenin also show anti-chondrogenic activity during joint formation [66, 67]. Recent data generated during chondrogenic differentiation of mesenchymal stem cells have shown that TGF-β3 treatment combined with β-catenin activation can circumvent the problem of hypertrophy induction and results in a more stable chondrogenic phenotype than the induction with TGF-β3 alone [68]. Therefore, it is tempting to speculate that treatment with other anti-chondrogenic stimuli during chondrogenic differentiation will result in the generation of fully functional and non-hypertrophic chondrocytes. Another way of achieving full differentiation without characteristics of hypertrophy is to force continuous expression of the master regulator SOX9. Studies in growth plate chondrocytes have shown that permanent induction of SOX9 inhibits terminal differentiation and hypertrophy by repression of the transcription factor RUNX2 [69, 70]. RUNX2 and RUNX3 are the pivotal inducers of type X and type I collagen as well as osteocalcin and MMP13 [71–73]. These markers are predominantly expressed in hypertophic chondrocytes present in cartilage affected by OA. Likewise the SOX9 RUNX2 interaction is another major pathway able to control onset of hypertrophy in chondrocytes and may represent an important signaling pathway for therapeutic intervention in OA. A schematic for growth factor induced signaling and its molecular consequences are shown in Fig. 1.

Scaffolds Support Chondrogenic Differentiation

Chondrogenic differentiation is greatly enhanced when cells are grown at high densities in so called pelleted three dimensional micromass cultures [46]. These culture conditions mimic in vivo joint development where mesenchymal cell condensation precedes the induction of chondrogenesis and extracellular matrix molecules like type I collagen and type II collagen are produced. For clinical applications self assembled pellet cultures are difficult to process. Because of this, cells are most frequently grown and differentiated on various scaffolds, which exhibit biomechanical stability and aid in the construction of artificial tissue. Commonly used biomaterials are either of natural origin like alginate, agarose, hyaluron acid and collagen or of synthetic origin like polyethylene glycol and poly-lactic-co-glycolic acid. Human amniotic fluid cells have so far been differentiated in three dimensional pellets and in hyaluronan based hydrogels [60–62]. Ovine amniotic fluid cells have been differentiated in biodegradable polyglycolic acid scaffolds and in synthetic polyglycolic acid [58, 74]. In general scaffolds provide a niche, which favors chondrocytes ECM production and viability. It aids in integrating at the site

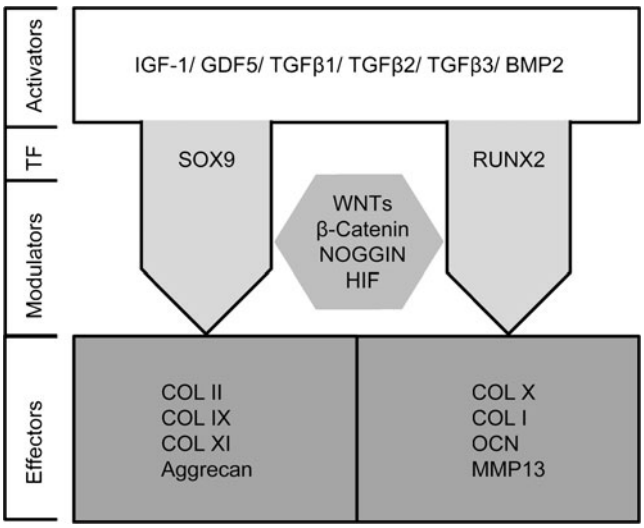

Fig. 1 Simplified signaling network controlling chondrocyte fate decision. Stem cells, in appropriate cell culture conditions, initiate chondrogenic differentiation after stimulation with TGFβ family members like GDF5, TGFβ1, TGFβ2, TGFβ3 and BMP2 in combination with IGF-1. Upregulation of the transcription factor SOX9 leads to functional chondrocyte differentiation, evidenced by collagen type 2, 9, 11 and aggrecan production. Upregulation of the transcription factor RUNX2 leads to chondrocyte hypertrophy, evidenced by collagen type 10, 1, osteocalcin and MMP13 production. Signaling pathways controlled by WNTs, β-Catenin, NOGGIN and HIF are able to either positively or negatively modulate chondrogenic differentiation. TF=transcription factors

of articular defect and should therefore also be biodegradable. Future applications of AFS cells will strongly depend on the correct choice of scaffolds for cartilage engineering.

Impact of Hypoxia on Chondrogenic Differentiation

Due to the avascular nature of articular cartilage, oxygen levels from the superficial zone to the calcified zone gradually decrease. It has also been shown that low oxygen tension enhances the differentiation process of human mesenchymal stem cells and of human articular cartilage cells, whereas in AFS cells, as far as we know, the impact of hypoxia on the chondrogenic potential has not been evaluated [75, 76]. Molecular studies of isolated human articular chondrocytes as well as of mouse limb bud mesenchyme revealed that hypoxic treatment results in SOX9 expression and that up regulation of HIF1α (hypoxia-inducible factor) and HIF2α is essential for SOX9 expression [77, 78]. Interestingly, chondrocyte hypertrophy, which is also detected during OA, is marked by extensive VEGFA production. VEGFA release leads to increased vascularisation in the affected organ area accompanied by increased mineralization. This generation of osteophytes during progression of OA has been shown to occur in the knee joint of rabbits induced to form OA by anterior cruciate ligament transection [79]. Recently it was also shown that hypoxia inhibits upregulation of RUNX2 and it was demonstrated that mesenchymal stem cells failed to differentiate into osteoblasts under hypoxic conditions because of the upregulation of HIF1α and TWIST [80]. Therefore reduced oxygen tension is beneficial for chondrogenic differentiation and for the maintenance of a stable chondrogenic phenotype and VEGFA production has to be avoided since it would result in endothelial cell activation.

Role of Autophagy for Chondrocyte Maintenance

During starvation and stress periods autophagy can protect cells by turnover of intracellular organelles and molecules. In articular cartilage autophagy plays an important role, since it protects chondrocytes from age related cell death and preserves homeostasis within the tissue [81]. During aging-related intracellular changes, biochemical stimuli as well as mechanical stress can lead to OA and during this process the competence to undergo autophagy is lost [82]. Therefore autophagy protects from aging-related cell death and probably it is also an important process during chondrogenic differentiation. Rapamycin, a well known inhibitor of mTORC1, has been shown to reduce the severity of experimentally induced OA in mice by activating autophagy [83]. The authors show that rapamycin treatment resulted in a significant increase in the number of chondrocytes in articular cartilage affected by OA. Furthermore, rapamycin treatment decreases the amount of ADAMTS-5, a major aggrecan degrading enzyme, and it also reduces secretion of IL-1β, an inflammatory cytokine. The authors also speculate that rapamycin treatment results in a reduction of reactive oxygen species and that therefore also aging-associated pathologies are suppressed. This effects can be attributed to mTOR modulation, which is the key regulator of cell growth and proliferation, and which among other processes also controls autophagy, HIF1α expression and ROS production [84–86]. Indeed several independent studies have shown that the mTOR pathway represents a major signaling pathway able to contribute to chondrocyte differentiation and homeostasis [87–89].

Conclusions and Future Perspectives

AFS cells represent a novel and promising cell type for regenerative, cell based therapies. The chondrogenic

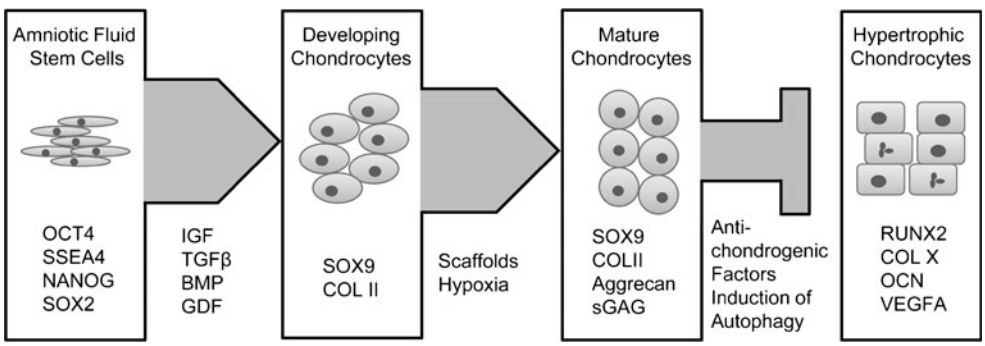

Fig. 2 Differentiation of amniotic fluid stem cells to chondrocytes. Combined growth factor signaling initiates cell condensations and the onset of chondrogenic differentiation in former pluripotent amniotic fluid stem cells. The use of scaffolds and the presence of hypoxic conditions further improve extracellular matrix production and contribute to the transition of developing chondrocytes to a mature chondrocyte state. Onset of hypertrophy has to be avoided since it would result in reduction of functional chondrocytes and in focal calcification of joint cartilage. Strategies to prevent hypertrophy and to sustain a stable chondrocyte phenotype include treatment with anti chondrogenic factors in a time depend fashion and the induction of autophagy. sGAG=sulfated glycosaminoglycans

potential of AFS cells has been shown, although the molecular parameters controlling the differentiation process are not well defined. Hence, differentiation efficiencies vary considerably and need further improvement. For therapeutic approaches large scale production and high quality of chondrogenic cells as well as thereof derived matrix are of paramount importance. Effective in vitro differentiation protocols need to recapitulate the spatio-temporal aspects of chondrocyte differentiation during development and, at the same time, inhibit the onset of chondrocyte hypertrophy. Ways to achieve this are summarized in Fig. 2 and include: I) Treatment with growth factors only for specific time periods, like for example incubation with TGF-β only at early phases of chondrocyte development, where cell condensation and ECM production takes place. II) Combinations of pro and anti-chondrogenic treatment regiments in a defined ratio and in a time dependent fashion. This will yield the beneficial effects of chondrogenic stimulation, while preventing the end stage maturation effects of hypertrophy. III) The use of scaffolds substantially increases differentiation efficiencies and fosters the formation of cartilage-like tissue. At the same time three-dimensional cultivation limits oxygen supply and therefore also contributes to enhanced differentiation. IV) The induction of autophagy is able to prevent ageing related degenerations. Therefore, novel compounds, like rapamycin derivatives, which stimulate autophagy with only little side effects can be supplemented in late phases of differentiation to prevent apoptosis and sustain a stable and mature chondrocyte phenotype. Further studies are needed to elucidate what combinations of treatments will yield the most in vivo like phenotype. A key point will be controlling the abundance of master regulators like the transcription factor SOX9, which is able to sustain the chondrogenic phenotype and RUNX2, which is predominantly expressed during chondrocyte hypertrophy. Taken together, since AFS cells can be biobanked, are genetically stable, immunoprivileged and non tumorigenic, these cells potentially resemble the most suitable cell source for cell based regeneration approaches in articular joint defects.

Conflicts of interest The authors declare no potential conflicts of interest.

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