Impaired remodeling phase of fracture repair in the absence of matrix metalloproteinase-2

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SUMMARY

The matrix metalloproteinase (MMP) family of extracellular proteases performs crucial roles in development and repair of the skeleton owing to their ability to remodel the extracellular matrix (ECM) and release bioactive molecules. Most MMP-null skeletal phenotypes that have been previously described are mild, thus permitting the assessment of their functions during bone repair in the adult. In humans and mice, MMP2 deficiency causes a musculoskeletal phenotype. In this study, we assessed the role of MMP2 during mouse fracture repair and compared it with the roles of MMP9 and MMP13. Mmp2 was expressed at low levels in the normal skeleton and was broadly expressed in the fracture callus. Treatment of wild-type mice with a general MMP inhibitor, GM6001, caused delayed cartilage remodeling and bone formation during fracture repair, which resembles the defect observed in Mmp9⁻/⁻ mice. Unlike Mmp9- and Mmp13-null mutations, which affect both cartilage and bone in the callus, the Mmp2-null mutation delayed bone remodeling but not cartilage remodeling. This remodeling defect occurred without changes in either osteoclast recruitment or vascular invasion of the fracture callus compared with wild type. However, we did not detect changes in expression of Mmp9, Mmp13 or Mt1-Mmp (Mmp14) in the calluses of Mmp2-null mice compared with wild type by in situ hybridization, but we observed decreased expression of Timp2 in the calluses of Mmp2⁻/⁻, Mmp9⁻/⁻ and Mmp13-null mice. In keeping with the skeletal phenotype of Mmp2-null mice, MMP2 plays a role in the remodeling of new bone within the fracture callus and impacts later stages of bone repair compared with MMP9 and MMP13. Taken together, our results indicate that MMPs play unique and distinct roles in regulating skeletal tissue deposition and remodeling during fracture repair.

INTRODUCTION

Matrix metalloproteinase-2 (MMP2) belongs to a large family of zinc-dependent enzymes that degrade the structural and non-structural components of the extracellular matrix (ECM) (McCawley and Matrisian, 2001; Mott and Werb, 2004), thus regulating many processes, including embryonic development, tissue repair and tumorigenesis (Egeblad and Werb, 2002; Page-McCaw et al., 2007; Parks et al., 2004). Inactivating mutations in the MMP2 gene in humans lead to skeletal disorders that are associated with bone loss and joint erosion, or multicentric osteolysis with arthritis (MOA) syndrome (Martignetti et al., 2001). Mice lacking Mmp2 exhibit attenuated features of the human MOA phenotype, characterized by progressive loss of bone mineral density, articular cartilage destruction, and abnormal long bone and craniofacial development (Inoue et al., 2006; Mosig et al., 2007). Detailed analysis of Mmp2⁻/⁻ mice revealed that MMP2 acts at the level of osteoclasts and osteoblasts, as well as in maintaining osteocytic networks necessary for osteocyte function and survival. The involvement of MMP2 in human skeletal disease and the overall mild phenotype of Mmp2⁻/⁻ mice prompted us to address the role of MMP2 in fracture repair.

Although most MMPs share similar characteristics in terms of enzymatic activities and substrate specificity, their functions in bone biology are distinct. Indeed, the lack of Mmp9, Mt1-Mmp (Mmp14) or Mmp13 during skeletal development causes growth plate abnormalities in long bones and impaired bone formation that differ from the Mmp2⁻/⁻ phenotype (Holmbeck et al., 1999; Stickens et al., 2004; Vu et al., 1998; Zhou et al., 2000). However, some aspects of the Mmp2⁻/⁻ and Mt1-Mmp⁻/⁻ phenotypes are comparable, with both enzymes being involved in osteocytogenesis (Holmbeck et al., 2005). The analysis of Mmp9⁻/⁻ and Mmp13⁻/⁻ mice during bone repair revealed that each of these enzymes acts on different cell types to produce comparable phenotypes. We reported that MMP9 that is released by osteoclasts in specific sites of the fracture callus regulates the angiogenic switch and cartilage removal during repair via endochondral ossification. By contrast, MMP13 operates at the level of the cartilage and bone matrices to prepare for their proper remodeling in the fracture callus (Behonick et al., 2007; Colnot et al., 2003). These results provided functional evidence that the role of MMPs during skeletal development is recapitulated during skeletal repair. Although members of the MMP family are likely to be essential for the regenerative process, the functions of each individual MMP have not been completely elucidated. In the present study, we examined the role of MMP2 in the process of endochondral ossification during fracture repair and investigated redundancies among MMP family members.

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RESULTS
Expression of Mmp2, Mmp9, Mmp13 and Mt1-Mmp during fracture repair

MMPs play important roles during bone formation via endochondral ossification. This process requires the deposition of a cartilage matrix, which is remodeled and replaced by bone in part via the action of MMPs. Because non-stabilized tibial fractures heal via endochondral ossification, we chose this model to compare expression patterns of MMPs. Mmp2 is expressed at low levels in uninjured bone, including in osteocytes (data not shown) (Inoue et al., 2006; Mosig et al., 2007). Low levels of Mmp2 expression were detected as early as day 3 post-fracture at the fracture site and in the surrounding soft tissues immediately adjacent to the fracture site (Fig. 1C). Mmp2 expression was low and diffuse compared with the highly localized expression of Mmp9 in osteoclasts (Fig. 1D) (Colnot et al., 2003), Mmp13 in the activated periosteum (Fig. 1E) (Behonick et al., 2007), and Mt1-Mmp signal in the activated periosteum and surrounding soft tissues (Fig. 1F). By day 6, Mmp2 expression was still diffuse (Fig. 2D) but, compared with day 3, was stronger in areas of the callus in which cartilage and bone form, as shown by adjacent sections stained with collagen type 1 and collagen type 2 in situ probes (Fig. 2A-C). This pattern differed from the Mmp9 expression pattern in osteoclasts (Fig. 2E,F), and Mmp13 expression in cartilage and bone (Fig. 2G). Diffuse expression of Mt1-Mmp (Fig. 2H) and the MMP activator basigin [Bsg; also known as extracellular matrix metalloproteinase inducer (EMMPRIN); Fig. 2I] were also found in areas that overlapped with Mmp2 expression. At day 10, when the callus comprises a large amount of cartilage tissue surrounding by areas of new bone, Mmp9 expression was still detected at low levels throughout the callus (Fig. 3D). On adjacent sections, Mmp9 expression was confined to the chondro-vascular junction (Fig. 3E), Mmp13 expression was high in hypertrophic cartilage and bone (Fig. 3F), Mt1-Mmp expression was high at the chondro-vascular junction but low in cartilage and bone (Fig. 3G), and Bsg expression was low throughout the callus (Fig. 3H). Interestingly, the MMP inhibitor Timp2 was also expressed in most areas of the callus, with a stronger signal observed at the junction of cartilage and bone, at which several MMPs are highly expressed (Fig. 3I).

Effects of the general MMP inhibitor GM6001 on fracture repair

Given the presence of multiple MMPs in the fracture callus, we first assessed the total contribution of MMPs on bone repair by applying the MMP inhibitor GM6001 directly into the fracture callus of wild-type mice. The treatment was applied during the early soft callus phase of repair (days 6-9). By day 10, the proportions of cartilage and bone were not significantly different between treated and control calluses (Fig. 4). GM6001 affected the initial hard callus phase of repair. Healing was delayed and marked by a significant increase in the proportion of cartilage within the callus at day 14 and a significant decrease in the proportion of bone (Fig. 4).

Delayed bone remodeling in Mmp2-null mutant fracture calluses

When we created non-stabilized tibial fractures in Mmp2−/− mice, we observed a delay in bone remodeling as shown by representative histological stains (Fig. 5A) and histomorphometric analyses (Fig. 5B). The callus volume was increased at day 10 but not at days 14 and 21 in Mmp2−/− mice compared with wild-type counterparts (Fig. 5B). The total cartilage volume and the proportions of cartilage in the callus were also unchanged in Mmp2−/− compared with wild-type mice (Fig. 5B). At day 10, the total bone volume and proportion of bone in the callus were not significantly different between Mmp2−/− and wild-type mice. By days 14 and 21, we observed an increase in the total bone volume and the proportion of bone in Mmp2−/− compared with wild-type mice, indicating a delay in bone remodeling in Mmp2−/− mice.

To investigate the cellular bases of this phenotype, we performed cellular and molecular analyses on representative sections through the fracture calluses at various time points. We observed normal osteoclast recruitment as indicated by tartrate-resistant acid phosphatase (TRAP) staining at day 10 post-fracture (Fig. 6A,E). Degradation of the cartilage matrix, as shown by antibody staining for the DIPEN epitope (an epitope within aggrecan that is exposed specifically upon MMP cleavage), revealed no changes in the state of proteoglycan cleavage in Mmp2−/− calluses compared with those in wild type (Fig. 6B,F). Similarly, blood vessel invasion assessed via platelet endothelial cell adhesion molecule (PECAM) immunostaining was normal in Mmp2−/− fracture calluses (Fig. 6C,G) and expression of the angiogenic factor Vegf in hypertrophic cartilage was similar in Mmp2−/− calluses compared with wild type. Given that the absence of Mmp2 affected mostly the bone compartment of the callus, we concentrated our expression analyses on bone but found no changes in the expression pattern of Mmp2 −/− mice.
osteopontin (Fig. 6J,N), collagen type 1 (Fig. 6K,O) and osteocalcin (Fig. 6L,P) at day 14 post-fracture.

Analysis of functional redundancy among MMP family members

The Mmp2–/– fracture repair phenotype differed from the more severe bone repair phenotypes that we observed previously in Mmp9–/– and Mmp13–/– mice. We thus investigated whether MMP2 loss was compensated for by other MMPs that are expressed in the callus. When we compared Mmp2–/– and wild-type calluses, we did not observe qualitative differences in the expression profiles or expression levels of Mmp9, Mmp13 or Mt1-Mmp (Fig. 7A-E,G-K). However, we observed a decrease in Timp2 expression in the callus of Mmp2–/– mice compared with wild type (Fig. 7F,L). Likewise, Mmp2, Mmp13 and Mt1-Mmp expressions were unchanged in Mmp9–/– calluses compared with wild-type calluses, but Timp2 exhibited a decrease in the intensity of in situ hybridization signal (Fig. 7M-R). The expression of Timp2 was also decreased in Mmp13–/– calluses compared with wild-type calluses, whereas the expressions of Mmp2, Mmp9 and Mt1-Mmp were similar to wild type (Fig. 7S-X). These results thus indicate that the absence of Mmp2 might not impact the expression of other MMPs in cartilage and bone, suggesting that normal expression levels might be sufficient to partially compensate for the lack of Mmp2.

DISCUSSION

Repair of the skeleton after traumatic injury is a very dynamic process involving multiple cell types and tissues. Healing is initiated by an inflammatory response followed by the recruitment and differentiation of skeletal stem cells, the production of cartilage and bone matrices, and finally remodeling of the matrix. In many aspects, the stages of bone regeneration resemble embryonic skeletal development. Bone can form either via intramembranous ossification defined by the direct formation of bone or via endochondral ossification defined by the replacement of cartilage matrix by bone. In endochondral ossification, the intermediate stage of cartilage deposition is essential to support ossification and requires extensive control of ECM deposition and remodeling. Furthermore, cartilage maturation and removal are synchronized with the invasion of blood vessels that support cartilage replacement and ossification. Coordinating the dynamic relationship between ECM deposition, degradation and angiogenesis depends on the interaction of numerous growth factors and signaling molecules as well as matrix degrading enzymes. In this study we assessed the role of MMP2 during fracture repair via endochondral ossification, compared with those of MMP9 and MMP13. The latter two MMPs were previously described to be key regulators of cartilage and/or bone remodeling in the fracture callus. Unlike the Mmp9–/– and Mmp13–/– mutations, which affected both cartilage and bone tissues during fracture repair, the Mmp2–/– mutation affected only the bone compartment of the fracture callus.

MMP2 does not impact cartilage and bone deposition during the soft callus phase of repair

We observed that Mmp2 was expressed throughout the fracture healing process starting as early as day 3 post-injury, which coincides with the inflammatory phase of repair. Shortly after bone injury, inflammatory cells invade the fracture site and produce many factors, including cytokines and MMPs. Cytokines can stimulate MMP activation, which in turn might facilitate the migration of...
inflammatory cells and the initiation of the repair process (Parks et al., 2004). Although MMP2 plays a role in regulating inflammation in other tissues and might be involved in the inflammatory response associated with fracture healing (Corry et al., 2004; Greenlee et al., 2006), its absence did not have a noticeable impact on the initial matrix deposition during the soft callus phase of repair. During the early stages of repair, we also observed a correlation between increased MMP expression, and particularly that of \textit{Mmp2}, and expression of \textit{Bsg}. BSG was discovered as a tumor surface molecule that stimulates surrounding fibroblasts to produce MMPs (Biswas et al., 1995); thus, MMP expression might be influenced by BSG during fracture repair.

Later, during the soft callus phase of repair, we observed diffuse and low expression levels of \textit{Mmp2}, which contrast with the strong and cell-type-specific expression patterns for \textit{Mmp9} in osteoclasts, \textit{Mmp13} in chondrocytes and osteoblasts, and \textit{Mt1-Mmp} in osteoclasts and osteoblasts. During bone development, \textit{Mmp2}–/– mice exhibit a mild phenotype with subtle growth delay, and develop some aspects of the MOA syndrome as the mice age (Inoue et al., 2006; Itoh et al., 1997; Mosig et al., 2007). At the cellular level, there is decreased osteoblast and osteoclast number in postnatal bones, and decreased osteoblastogenesis and osteoclastogenesis, in vitro (Mosig et al., 2007). However, our in vivo assessment of fracture calluses did not reveal decreased expression of bone markers or decreased recruitment of osteoclasts during the soft

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\caption{\textit{Mmp2} expression relative to other MMPs at day 10 post-fracture. (A) Safranin-O (SO) staining of wild-type callus tissues. Boxed area shows the transition between hypertrophic cartilage and bone, and is shown at higher magnification in B-L. (B-K) In situ hybridization on adjacent sections using (B) osteocalcin (Oc), (C) collagen type 10 (Col10), (D) \textit{Mmp2}, (E) \textit{Mmp9}, (F) \textit{Mmp13}, (G) \textit{Mt1-Mmp}, (H) \textit{Bsg} and (I) \textit{Timp2} antisense mRNA probes, (J) \textit{Mmp2} sense mRNA probe, and (K) \textit{Vegf} antisense mRNA probe. (L) PECAM immunostaining on an adjacent section illustrates the chondro-vascular junction. Scale bars: 1 mm (A); 200 μm (B-L).}
\end{figure}

\begin{figure}[h]
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\caption{The MMP inhibitor GM6001 delays cartilage remodeling and bone formation during fracture repair. Histomorphometric analyses at days 10 (d10) and 14 (d14) post-fracture of cartilage volume as a proportion of total callus volume (CV/TV) and bone volume as a proportion of total callus volume (BV/TV) in wild-type mice treated with GM6001 or carrier (carboxymethylcellulose; CMC) (n=3 per group; *P<0.05, Student’s t-test).}
\end{figure}
and hard callus phases of repair in Mmp2−/− mice. Taken together, these observations made during bone development, bone growth and in adult mice suggest that MMP2 action on skeletal lineages is not crucial during the early stages of repair; rather, MMP2 affects later stages of osteoblast and osteocyte differentiation during the remodeling phase of repair.

**Cellular basis for the bone remodeling phenotype in Mmp2-null mutant mice**

Consistent with the late onset of bone anomalies in Mmp2−/− mice, we observed a remodeling phenotype in Mmp2−/− mice during fracture repair. MMP2 is expressed in multiple cell types within the fracture callus, and could potentially regulate callus remodeling at many levels. For example, MMP2 in chondrocytes and osteoblasts could contribute intrinsically to cartilage and/or bone matrix turnover and remodeling. Although MMP2 might also be produced by osteoclasts that accumulate in the callus at sites of new bone formation, it might not play a significant role in this location. Our previous work illustrates that MMP9 acts mainly at the level of osteoclasts to regulate callus remodeling, whereas MMP13 acts at the levels of chondrocytes and osteoblasts. However, unlike Mmp9−/− and Mmp13−/− mutants, Mmp2−/− mutants did not exhibit delayed cartilage remodeling, but only exhibited bone remodeling defects. In addition, the delay in cartilage remodeling in Mmp9−/− mutants was closely associated with a delay in vascular invasion of the fracture callus. Although MMP2 has a role in angiogenesis in other systems (Kato et al., 2001), we did not detect notable differences in the infiltration of the callus by blood vessels in Mmp2−/− mice. Given that the transition from cartilage to bone depends on the coordinated degradation and vascular invasion of hypertrophic cartilage, this observation further supports the contention that MMP2 is not involved in this transition.

Bone remodeling in the late stages of repair remodeling is essential to restore the structural and mechanical properties of the broken bone. MMP2 might support the reestablishment of the mechanical properties of the bone matrix at late stages of repair. Thus, the delayed bone remodeling in Mmp2−/− calluses is probably due to MMP2 function at the level of the bone matrix itself. Interestingly, osteolytic lesions in adult Mmp2−/− mice are linked to the role of MMP2 in maintaining osteocytic processes (Inoue et al., 2006). During repair, MMP2 might also regulate osteocyte function during the late phase of repair. Further studies will be required to identify the specific MMP2 targets and/or substrates in various physiological conditions and to understand the molecular bases for distinct MMP phenotypes.
Do MMPs exert overlapping functions during fracture repair?
MMPs play multiple roles during the development and growth of the skeleton, as indicated by the divergent phenotypes of each individual MMP-null mutant. At the same time, overlapping functions have been uncovered by the amplified phenotypes observed in the combined absence of two MMPs. For example, the growth plate phenotype of Mmp9-Mmp13 double mutants is very severe compared to the growth plate phenotype of either Mmp9- or Mmp13-null mutants (Stickens et al., 2004; Vu et al., 1998). Compensatory mechanisms are also revealed by the fact that some MMP-null phenotypes resolve overtime. These functional compensations might occur within the MMP family or through the action of other enzymes, allowing deficits in bone formation to be overcome as the mutant mice mature.

Following bone injury, repair requires a rapid and massive production and remodeling of skeletal tissues. It is not surprising that the action of multiple MMPs with potential overlapping functions is again required during the regenerative process. These potential compensations among MMP family members were not detectable at the molecular level through upregulation of MMP gene expression. MMP activity in the fracture callus might not be regulated at only the RNA level but also at the protein level. MMP function can be regulated via tissue inhibitors of metalloproteinases (TIMPs) (Page-McCaw et al., 2007; Geoffroy et al., 2004). For example, MMP2 is a known target of TIMP2 and MT1-MMP (Cowell et al., 1998; Howard et al., 1991). Indeed, we observed a decrease in Timp2 expression, suggesting a possible compensation of Mmp2, Mmp9 and Mmp13 inactivation via the downregulation of TIMP2.
of endogenous MMP inhibitors in the fracture callus. These compensations are only partial: MMP-null mutations observed so far impact various stages of repair, delaying bone from accomplishing repair in a timely manner and reaching its preexisting uninjured state.

Due to the fact that the MMP family comprises more than 20 enzymes, we assessed the overall functions of the MMP family by applying a general MMP inhibitor, GM6001, directly at the fracture site. GM6001 caused delayed cartilage remodeling and bone formation, a phenotype that clearly differs from the Mmp2–/– phenotype but mimics the phenotype observed in Mmp9–/– mice. These results indicate that MMP9 acts earlier than MMP2 during fracture repair and is a limiting factor during the replacement of cartilage by bone in the callus. This is probably due to the unique substrate specificity of MMP9, and in particular the MMP9-dependent release of VEGF from hypertrophic cartilage (Lee et al., 2005; Ortega et al., 2005; Ortega et al., 2010).

In conclusion, our results on the role of MMP2 in bone remodeling during fracture repair show that it acts at later stages of repair compared with MMP9 and MMP13, revealing distinct functions of MMPs in bone repair. It is expected that MMPs could potentially be targets for the treatment of bone diseases and to accelerate skeletal repair.

METHODS
Non-stabilized tibial fractures and treatment with GM6001
All protocols were approved by the Institutional Animal Care and Use Committee. We used a well-established murine tibia fracture model to evaluate the role of MMP2 during bone repair. Adult 12- to 16-week-old wild-type and Mmp2–/– male mice in the FVB/N background were anesthetized and a non-stabilized tibial fracture was created in the mid-diaphysis via three-point bending (Behonick et al., 2007; Colnot et al., 2003; Lu et al., 2005). Animals were given analgesics during the period of anesthesia and 24 hours post-surgery. Mice receiving non-stabilized fractures typically recover in less than 24 hours and do not show signs of pain and discomfort when receiving the proper dose of anesthesia and analgesia. Nevertheless, mice were monitored daily in order to deliver additional doses of analgesics as needed. Fracture calluses were collected at days 10, 14 and 21 post-fracture (n=5 per group). For expression analyses, non-stabilized fractures were created in wild-type mice and the fracture calluses were collected at days 3, 6 (n=3 per group), 10 and 14 (n=5 per group) post-fracture. Non-stabilized fractures were created in Mmp9–/– and Mmp13–/– mice, and the fracture calluses were collected at day 14 post-fracture for expression analyses (n=3 per group). The MMP inhibitor GM6001 was as described previously (Ortega et al., 2010). GM6001 (20 mg/ml in 4% carboxymethylcellulose; Sigma) was injected directly into the fracture site of wild-type anesthetized mice at days 6, 7, 8 and 9 post-fracture (0.6 mg per injection; n=3 per group). The fracture calluses were collected at days 10 and 14 post-fracture for histomorphometric analyses (n=3 per group).
**RESULTS**

In this study, the authors show that Mmp2 is broadly expressed in the fracture callus at all stages of repair, and displays a distinct expression pattern compared with Mmp13 and Mmp9, which are specifically expressed in osteoblasts/chondrocytes and osteoclasts, respectively. The general MMP inhibitor GM6001 causes delayed cartilage remodeling and delayed bone formation during fracture repair, whereas Mmp2-null mutant mice only exhibit delayed bone remodeling. Impaired bone remodeling in Mmp2–/– mice is not associated with impaired osteoclast recruitment and vascular invasion of the fracture callus. The absence of Mmp2 is not compensated for by overexpression of Mmp9, Mmp13 or Mmp1 (Mmp14) in the calluses of Mmp2-null mice, although there is decreased expression of the endogenous MMP inhibitor Timp2, indicating that MMP activity and callus remodeling are regulated at multiple levels.

**Implications and future directions**

These results show that Mmp2 plays a role in the remodeling phase of fracture repair and acts at later stages than Mmp9 and Mmp13. Members of the MMP family have distinct functions during bone repair. Some MMPs might be better exploited to improve bone remodeling. However, owing to the multiple functions of MMPs in skeletal tissues, further in vivo studies are necessary before therapeutic targeting of these molecules can be considered.

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**Histomorphometric, cellular and molecular analyses**

Fracture calluses were collected at various time points post-fracture as indicated above, fixed, paraffin embedded and sectioned (n=5 per group). Safranin-O and Trichrome staining were used to quantify cartilage, bone formation and bone volume via histomorphometry (Behonick et al., 2007; Colnot et al., 2003; Lu et al., 2005). In situ hybridization using 35S-labeled antisense riboprobes was performed to assess Mmp2, Mmp9, Mmp13, Mti-Mmp, Timp2, Vegf, Bsg, collagen type 1, osteocalcin and osteopontin expression on adjacent sections (Behonick et al., 2007; Colnot et al., 2003). To confirm the specificity of Mmp2 in situ hybridization signal, the Mmp2 sense riboprobe was also used on adjacent sections (Wiseman et al., 2003). TRAP staining was performed to identify osteoclasts, DIPEN immunostaining to identify aggrecan cleavage and PECAM immunostaining to identify blood vessels (Behonick et al., 2007; Colnot et al., 2003). To label blood vessels, PECAM immunostaining was performed on adjacent sections; to label matrix resorbing cells, MMP9 immunostaining was performed in conjunction with TRAP staining (Colnot et al., 2003).

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**COMPETING INTERESTS**

The authors declare no competing or financial interests.

**AUTHOR CONTRIBUTIONS**

S.L. performed experiments and data analysis, and prepared the manuscript; E.H. performed experiments and data analysis, and edited the manuscript; R.D. and D.B. performed experiments and data analysis; Z.W. contributed reagents and edited the manuscript; T.M. and R.M. developed the approach and edited the manuscript; and C.C. developed the approach, performed data analysis and prepared the manuscript.

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