Analysis of DNA methylation reveals a partial reprogramming of the Müller glia genome during retina regeneration

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Upon retinal injury, zebrafish Müller glia (MG) transition from a quiescent supportive cell to a progenitor cell (MGPC). This event is accompanied by the induction of key transcription and pluripotency factors. Because somatic cell reprogramming during induced pluripotent stem cell generation is accompanied by changes in DNA methylation, especially in pluripotency factor gene promoters, we were interested in determining whether DNA methylation changes also underlie MG reprogramming following retinal injury. Consistent with this idea, we found that genes encoding components of the DNA methylation/demethylation machinery were induced in MGPCs and that manipulating MGPC DNA methylation with 5-aza-2′-deoxycytidine altered their properties. A comprehensive analysis of the DNA methylation landscape as MG reprogram to MGPCs revealed that demethylation predominates at early times, whereas levels of de novo methylation increase at later times. We found that these changes in DNA methylation were largely independent of Apobec2 protein expression. A correlation between promoter DNA demethylation and injury-dependent gene induction was noted. In contrast to induced pluripotent stem cell formation, we found that pluripotency factor gene promoters were already hypomethylated in quiescent MG and remained unchanged in MGPCs. Interestingly, these pluripotency factor promoters were also found to be hypomethylated in mouse MG. Our data identify a dynamic DNA methylation landscape as zebrafish MG transition to an MGPC and suggest that DNA methylation changes will complement other regulatory mechanisms to ensure gene expression programs controlling MG reprogramming are appropriately activated during retina regeneration.

repair | multipotent | Ascl1 | bisulfite sequencing | DNA sequencing

Induced pluripotent stem cells (iPSCs) can be generated through the forced expression of pluripotency factor genes, such as *Oct4*, *Klf4*, *Sox2*, *c-Myc*, *Lin28*, and *Nanog*, which are normally expressed in embryonic stem cells (ESCs) (1, 2). Pluripotency factor gene expression in ESCs and iPSCs is associated with chromatin that is in an "open" accessible state, whereas their repression in somatic cells is associated with less accessible, condensed chromatin (3). DNA methylation has a significant impact on chromatin structure (3). DNA demethylation of pluripotency factor promoter regions is correlated with increased expression during iPSC formation (4, 5). Similar epigenetic changes are seen in other cellular reprogramming events such as nuclear transfer (6), heterokaryon formation (7), and carcinogenesis (8).

Tissue regeneration provides another avenue to study cellular reprogramming. Unlike mammals, zebrafish can regenerate multiple tissues, including the retina. During zebrafish retina regeneration, Müller glia (MG) reprogram to generate progenitor cells (MGPCs) capable of replacing all lost neural cell types (9–12). The role that MG play during this regenerative event can be roughly divided into three phases: the replication-independent transition of MG to a MPGC occurring by 2 d postinjury (dpi), the assymetric division and proliferative amplification of MGPCs (13) between 2 and 7 dpi, and the differentiation of these progenitors following 7 dpi (Fig. S1*A*). MGPC formation is accompanied by the increased expression of pluripotency factors (14) and other key signaling molecules (15–21). The induction of pluripotency genes, along with the finding that the putative cytidine deaminases Apobec2a and Apobec2b (Apobec2a,2b) are necessary for MGPC formation (22), is consistent with the idea that active modification of the DNA methylation landscape may underlie the reprogramming of MG to MGPCs.

To test this idea, we compared the methylation profiles of MG and MGPCs using reduced representation bisulfite sequencing (RRBS). This comprehensive analysis identified a changing DNA methylation landscape underlying MG reprogramming and progenitor formation. Surprisingly, unlike the reprogramming of fibroblasts to iPSCs, promoters of genes encoding pluripotency factors were hypomethylated in MG and remained so in MGPCs. Furthermore, we were surprised to find that Apobec2a,2b had little impact on site-specific DNA demethylation. This analysis of the DNA methylation landscape as MG reprogram to an MGPC will serve as an important resource for understanding the function of DNA methylation during cellular reprogramming and tissue regeneration.

Results

Modulators of DNA Methylation Are Transcriptionally Regulated During MG Reprogramming. Previous studies suggested that many of the genes encoding regulators of DNA methylation were induced in

Significance

Retinal injury in zebrafish stimulates Müller glia (MG) to undergo a reprogramming event that transitions their identity from quiescent supportive cells to multipotent progenitors capable of repairing the damaged retina. Understanding the mechanisms underlying this reprogramming event may provide insights for stimulating retina regeneration in mammals. Here we report an epigenetic analysis of zebrafish MG, monitoring changes in their genomic methylation levels as they reprogram and generate progenitors. Although we find a changing DNA methylation landscape, our data also suggest that a basal methylation program may facilitate this transition. Interestingly, certain aspects of this basal methylation program are shared with mammals, suggesting they too may acquire progenitor properties under appropriate circumstances.

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the injured retina (22). To assess whether these genes were specifically induced in MGPCs, we used FACS to isolate MG from uninjured *gfap:gfp* transgenic fish retinas and MGPCs from injured *1016 tuba1a:gfp* transgenic fish retinas at 4 dpi (Fig. S1 *B* and *C*) (16). We have shown previously that mechanical lesion with a 30-gauge needle results in the activation of MG only at the site of injury and that *1016 tuba1a:gfp* transgenic fish specifically label MGPCs at 4 dpi (16). Quantitative real-time PCR showed increased expression of genes associated with DNA demethylation, such as *gadd45β*, *gadd45βl*, *gadd45γ*, *gadd45γl*, *apobec2a*, *apobec2b*, *tdg*, and *tet3* in MGPCs (Fig. 1 *A*–*C*). Of those associated with DNA methylation, we found increases of *dnmt1*, *dnmt4*, *dnmt5*, and *dnmt7* (Fig. 1 *A* and *D*). Expression analyses using mRNA prepared from whole retina likewise showed injury-dependent induction of these *dnmt* mRNAs (Fig. S1 *D* and *E*). Basal expression levels of these and other DNA methylation-associated genes were observed in quiescent MG (Fig. 1*A*). These results suggest that the regulation of DNA methylation may be important for MGPC formation.

Dnmt Inhibition Perturbs MG Reprogramming and MGPC Proliferation, Migration, and Differentiation. Treatment of zebrafish embryos with 5-aza-2′-deoxycytidine (5-dAza) has been shown to induce global DNA hypomethylation (23) and alter gene expression (24). To investigate whether DNA methylation regulates MGPC formation and properties, we treated injured *1016 tuba1a:gfp* transgenic fish daily with 5-dAza. Because the inhibition of Dnmts by 5-dAza requires its incorporation into DNA, de novo DNA methylation events occurring before the beginning of the first DNA replication will not be perturbed. GFP expression in these fish report MG reprogramming to a MGPC (9). Interestingly, 5-dAza treatment caused a dramatic increase in GFP expression in MGPCs, whose identity was confirmed by colabeling with PCNA (Fig. 2*A* and Fig. S2 *A* and *B*). However, treatment with 5-dAza also reduced MGPC proliferation (Fig. 2 *B* and *C*) and perturbed the localization of PCNA-positive cells in the outer nuclear and ganglion cell layers (Fig. 2 *B* and *D*). Although the majority of PCNA-positive cells at the site of injury likely derive from MGPCs, as indicated by their colocalization with GFP (Fig. S2 *A* and *B*), we cannot rule out the possibility that some of the reduced proliferation in the outer nuclear layer is due to a decrease in rod progenitor proliferation

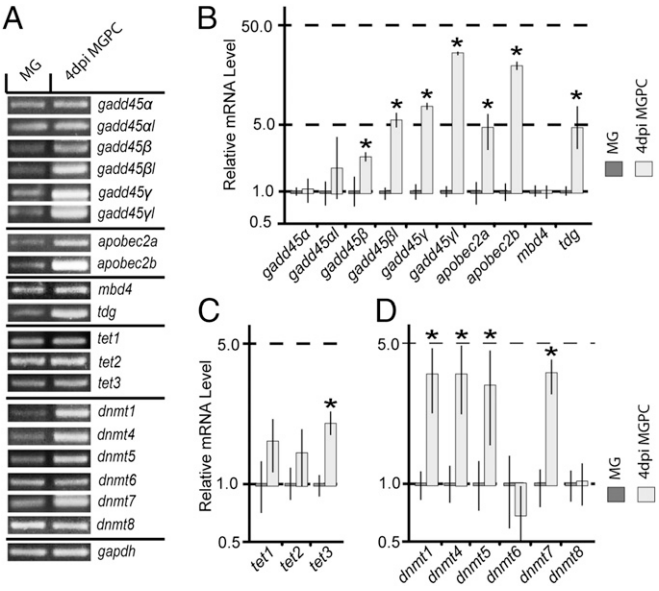

Fig. 1. Transcript levels of potential regulators of DNA methylation are induced during MGPC formation. (*A*) RT-PCR gene expression comparisons of quiescent MG (*gfap:gfp*) and 4-dpi MGPC (*1016 tuba1a:gfp*) cell populations targeting genes correlated with the regulation of DNA methylation. (*B* and *C*) Quantification of potential DNA demethylase mRNAs in *A* by quantitative PCR. **P* < 0.02403. (*D*) Quantification of DNA methyltransferase mRNAs in *A* by quantitative PCR. **P* < 0.04117. Error bars represent standard deviation.

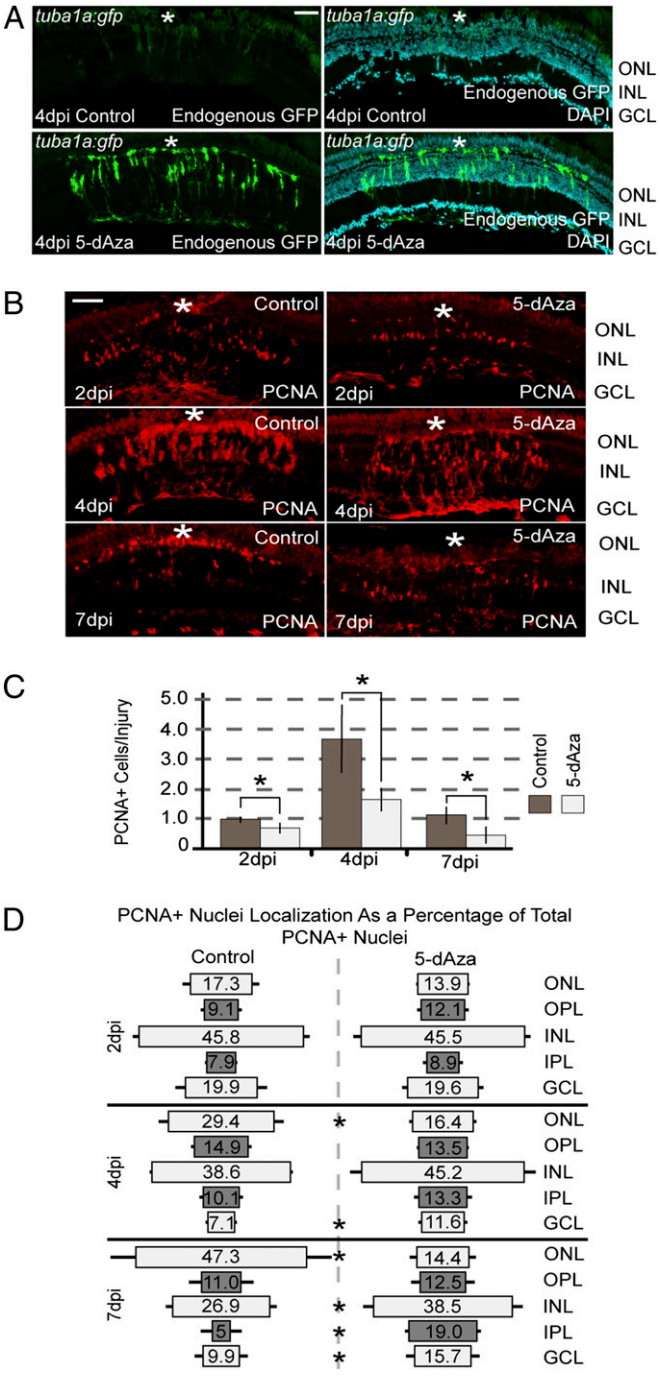

Fig. 2. Inhibition of Dnmts suggests that the regulation of DNA methylation is important for MG reprogramming. (*A*) Four-day postinjury *1016 tuba1a:gfp* transgenic fish treated with 5-dAza show increased levels of endogenous (unstained) GFP. (*B*) 5-dAza inhibits the generation and localization of PCNA-positive MG-derived progenitors. *, mark the injury site in *A* and *B*. (*C*) Quantification of PCNA-positive nuclei per injury site. The value is normalized to the value of the 2-dpi control. **P* < 0.02873. (*D*) Graphic depicting the localization of PCNA-positive nuclei within the retina at the indicated time points. 5-dAza perturbs the localization of PCNA-positive nuclei at 4 and 7 dpi. In *C* and *D* error bar extending from the box represent standard deviation. **P* < 0.04128. GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer; OPL, outer plexiform layer. (Scale bars, 50 μm.)

at the injury site. Treatment with 5-dAza did not seem to impact the number of MG responding to injury, as it did not alter the number of MGPC columns at 2 dpi when MG are just beginning to proliferate (Fig. S2*C*). In addition, 5-dAza perturbed the migration and differentiation of MGPC-derived progeny (Fig. S2 *D–G*). 5-dAza treatment without injury did not impact GFP expression or proliferation in *1016 tuba1a:gfp* transgenic fish (Fig. S2*L*).

To determine whether the effects of 5-dAza were due to a cytotoxic effect on MG, TUNEL assays were performed. 5-dAza treatment did not increase the absolute number of $TUNEL^+$ nuclei at the injury site at any time point analyzed (Fig. S2*H*). However, on rare occasions, we did note a *1016 tuba1a:gfp*, $GFP^+/TUNEL^+$ cell in the 5-dAza sample, but never in controls (Fig. S2*I*). These data, along with the fact that many MGPCs survive to 10 dpi (Fig. S2*F*), indicate that 5-dAza is only weakly cytotoxic to MGPCs. We then confirmed that 5-dAza actually caused hypomethylation of the MGPC genome by performing restriction PCR with McrBC, a methylation-sensitive endonuclease (Fig. S2 *J* and *K*). Together, these results suggest that DNA demethylation may activate genes, such as *tuba1a*, that are associated with MG reprogramming, and may also impact genes contributing to MGPC proliferation, migration, and differentiation.

Comparison of MG and MGPC DNA Methylation Landscapes at 4 Days Postinjury. To determine whether DNA methylation changes underlie the conversion of MG to MGPCs, we used RRBS to identify site-specific DNA methylation changes in these two cell types. For this analysis, MG were FACS-purified from uninjured *gfap:gfp* fish retinas and MGPCs were FACS-purified from injured *1016 tuba1a:gfp* fish retinas at 4 dpi. These fish have been bred to the same wild-type background for many generations, and we confirmed that they have highly similar backgrounds using in-depth SNP analysis (*SI Materials and Methods*).

Genomic DNA (gDNA) was isolated from MG and MGPCs and their methylation profiles were compared by RRBS (Fig. S3 and Table S1). The methylation levels of 611,434 individual cytosines within the CpG context were quantitatively compared between the two cell populations, and of those, 9,554 were differentially methylated bases (DMBs) (Fig. 3*A*). This represents a difference of 1.56% between the methylation profiles. Fifty-four percent and 46.0% of these DMBs were increasing and decreasing methylation, respectively (Fig. 3*B*). Validation of a subset of these DMBs was performed by restriction PCR after digesting MG and 4-dpi MGPC gDNA with the methylation-sensitive restriction enzyme HpaII (Fig. S4 *A–D*). Consensus sequences for methylated and demethylated sites were analyzed using 10 bp flanking the DMB, and no clear consensus was found outside of the centrally located CpG.

Interestingly, DMBs were not congregated within the genome, but were thinly dispersed along all chromosomes (Fig. S3*H*). Most DMBs, regardless of the direction of change in methylation, were localized to intergenic and intronic regions, whereas relatively few were localized to exonic and promoter regions (Fig. 3*C*). There was a dearth of DMBs localized to CpG islands and shores, suggesting that the bulk of the methylation changes are occurring in CpG-poor regions (Fig. 3*C*). Surprisingly, no DMBs were localized to the promoters of pluripotency factors and previously characterized retina regeneration-associated genes, suggesting that promoter methylation/demethylation was not involved in their regulation. This differs from previously studied reprogramming events that correlated pluripotency factor promoter region demethylation with increased gene expression (5–7).

Whereas DNA methylation predominantly occurs within the CpG context, non-CpG methylation can occur in the contexts of CHG and CHH (H represents A, C, or T). The methylation levels of 775,005 CHG and 1,554,382 CHH cytosines were quantitatively compared by RRBS, and of those, 440 and 635 were differentially methylated, respectively (Fig. S4 *J*, *K*, *M*, and *N*). This represents changes of only 0.057% and 0.041% in their levels, respectively. Like DMBs in the CpG context, CHG and CHH DMBs were also predominantly localized to CpG-poor intergenic and intronic regions (Fig. S4 *L* and *O*).

Promoter DMBs That Are Decreasing Methylation Are Correlated with Increases in Gene Expression. Because DNA methylation is thought to regulate gene transcription (25), we decided to further analyze the DMBs that are localized to promoter regions. Specifically, we sought to determine whether promoters with DMBs that are decreasing methylation show increased expression levels and vice versa. We analyzed 6,210 promoters by RRBS, and of those, 292 contained at least one DMB (4.7%) (Fig. S4*E*). Previous microarray expression data comparing MG and 4-dpi MGPC cell populations quantitatively compared the expression of 8,039 genes, of which 1,361 were increasing expression and 262 were decreasing expression in MGPCs (Fig. S4*F*) (17). Comparison of these datasets revealed little correlation between promoter DMBs with increasing methylation and gene expression. However, we did find a correlation between promoter DMBs with decreasing methylation and increased gene expression (Fig. 3*D* and Fig. S4*G*). To confirm these trends, the expression levels of a random subset of genes (whose promoters contained at least one DMB) were analyzed using cDNA prepared from FACS-purified MG and 4-dpi MGPCs (Fig. S4 *H* and *I*). These results support the correlations found in the RRBS–microarray comparison.

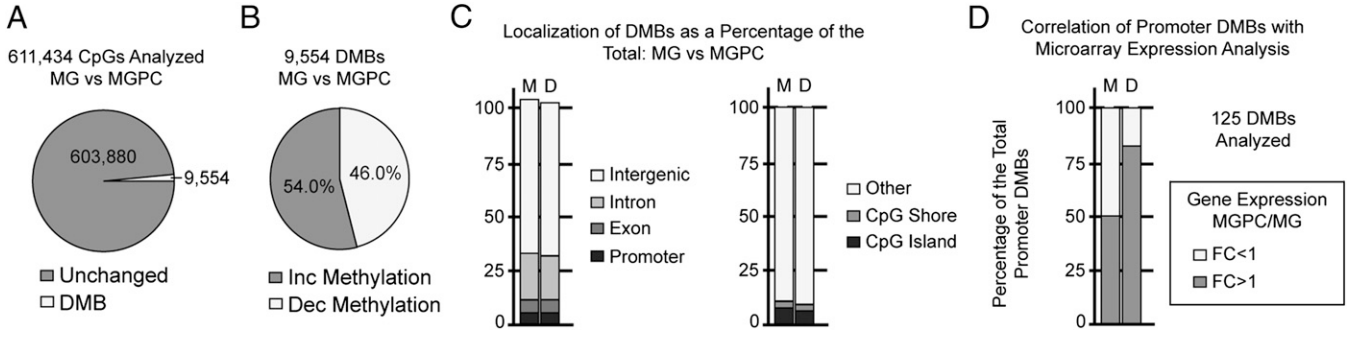

Fig. 3. Site-specific regulation of DNA methylation accompanies MG reprogramming. (*A–C*) RRBS comparison of quiescent MG (*gfap:gfp*) and 4-dpi MGPCs (*1016 tuba1a:gfp*). Quantification (*A*) and classification (*B*) of DMBs. (*C*) Localization of DMBs by type: D, demethylated; M, methylated (some DMBs fall into multiple classes, so the total exceeds 100%). Most DMBs are localized to intergenic, CpG-poor regions. (*D*) Correlation of RRBS promoter DMBs with previously obtained microarray gene expression data (16). Classification of DMBs by their localization to gene promoters increasing (FC > 1) or decreasing (FC < 1) expression during MG reprogramming. No correlation is seen between gene expression and DMBs that are increasing methylation (M), whereas a correlation is seen between increased gene expression and DMBs that are decreasing methylation (D). FC, fold change. Promoters were defined as 5 kb upstream and 1.0 kb downstream of the transcription start site.

Apobec2-Independent DNA Demethylation Predominates as MG Transition to MGPCs. We previously showed that Apobec2a,2b are required for MGPC formation and hypothesized that they participate in active DNA demethylation of the MG genome following injury (22). We sought to use RRBS to directly test whether they regulate DNA demethylation. Our RRBS dataset comparing MG and 4-dpi MGPCs cannot differentiate between DMBs arising passively (replication-dependent) and actively (replication-independent). The knockdown of Apobec2 proteins blocks MGPC proliferation, so analyzing their effects at 4 dpi may bias our results due to a block of passive DNA demethylation. Furthermore, because the knockdown of Apobec2a,2b blocks *1016 tuba1a:gfp* transgene induction, an alternate method was needed to purify MGPCs following knockdown.

To overcome these obstacles, we injected and electroporated control or *apobec2a,2b* lissamine-tagged antisense morpholino-modified oligonucleotides (MOs) into *gfap:gfp* transgenic fish and performed six additional lesions postelectroporation, resulting in the activation of MG across the entire retina. GFP and lissamine-positive cells were then isolated by FACS at 2 dpi (Fig. S5 *A* and *B*). This protocol resulted in the isolation of all MG that have MOs, not only those responding to injury (MG and MGPCs). To estimate this enrichment, GFP^+/$lissamine^+$ MGPCs were isolated from control MO-treated *1016 tuba1a:gfp* or *gfap:gfp* fish at 4 dpi, and *ascl1a* mRNA levels (a transcript restricted to MGPCs) were compared (Fig. S5*J*). This analysis indicates that ~60% of the GFP^+/$lissamine^+$-sorted cells from *gfap:gfp* fish are MGPCs.

At 2 dpi, MGPCs are just beginning their proliferative response (9), so the majority of DMBs identified by RRBS should result from active demethylation. RRBS libraries were prepared using 2-dpi MGPC populations treated with control or *apobec2a,2b* MOs (Fig. S5 *C–I* and Table S1). We first compared the methylation profiles of FACS-purified MG with 2-dpi MGPCs electroporated with control MOs (Fig. 4 *A–C* and Fig. S6 *A–D*). We analyzed a total of 360,448 CpGs and found 7,535 DMBs (Fig. 4*A*). Unlike at 4 dpi, the majority of DMBs at 2 dpi were decreasing methylation (73.6%) (Fig. 4*B*), but similar to 4 dpi, the DMBs were predominantly localized to CpG-poor promoter and intronic regions (Fig. 4*C*). In addition to changes in CpG methylation, 317 and 613 DMBs were found in the context of CHG and CHH, respectively (Fig. S6 *C* and *D*). These results suggest that DNA methylation is actively regulated during MGPC formation.

When we compared the methylation profiles of 2-dpi MGPCs from control and *apobec2a,2b* MO-treated retinas (Fig. 4*D* and Fig. S6 *L–R*), a total of 283,572 CpGs was analyzed and only 66 DMBs were found, a difference of only 0.023% (Fig. 4*D*). In addition, 63.6% of the DMBs identified were more demethylated following knockdown of Apobec2a,2b, opposite what one would expect if they were participating in DNA demethylation (Fig. S6*P*). Non-CpG methylation was largely unaffected, with no CHG DMBs and only one CHH DMB (Fig. S6 *N* and *O*). Methylation comparisons of MG and 2-dpi *apobec2a,2b* MO-treated MGPCs were very similar to those between MG and 2-dpi control MO-treated MGPCs (Fig. S6 *E–K*). These results strongly suggest that Apobec2 proteins do not participate in site-specific active DNA demethylation during MG reprogramming. When we compared the overall genomic methylation of these cell populations, we did see that the 2-dpi MGPC, *apobec2a,2b* MO-treated cell population was slightly more methylated than the 2-dpi MGPC, control MO-treated population, suggesting that Apobec2a,2b may directly or indirectly regulate global DNA methylation levels (Fig. S6*R*).

Pluripotency Factor and Regeneration-Associated Gene Promoters Are Hypomethylated in Quiescent MG. As mentioned previously, the promoter methylation levels of genes encoding pluripotency factors and select regeneration-associated genes remained unchanged upon MGPC formation. Surprisingly, when we looked at the basal (quiescent MG) promoter methylation of these genes (including *ascl1a*, *insm1a*, *hbegfa*, *lin28*, *sox2*, *oct4*, *nanog*, *c-mycb*, and *klf4*), we found that they were hypomethylated (Fig. 5*B*). When we compared RRBS basal promoter methylation levels with our microarray expression data, we found that genes highly induced in 4-dpi MGPCs tended to have lower than average promoter (Fig. 5*C*) and intragenic (Fig. 5*D*) methylation levels. We confirmed the basal hypomethylation of a number of these promoters by bisulfite (Fig. 5 *E* and *F*) and restriction (Fig. 5*H* and Fig. S7*A*) PCR. Slight discrepancies seen between RRBS, bisulfite PCR, and restriction PCR may be attributed to the regions targeted for analysis (outlined in Fig. 5*A*). Surprisingly, MG and non-MG had similar restriction PCR profiles (Fig. 5*H* and Fig. S7*A*). These results suggest that pluripotency factor and many regeneration-associated genes may be poised for activation in quiescent MG, although further work is needed to analyze other epigenetic marks. Thus, zebrafish MG may require only limited reprogramming, and may be more stem cell-like in nature than previously thought.

The regenerative capability of mammalian MG is very limited compared with zebrafish MG and is lost with age (26). We wondered whether this may be due to differences in methylation of regeneration-associated gene promoters. To assess whether the basal promoter methylation levels differ between zebrafish and mouse MG, we isolated mouse MG from uninjured retina by staining with an anti-GLAST PE antibody followed by FACS. MG enrichment was confirmed by PCR (Fig. 5*G*). When we analyzed the methylation levels of a limited number of mouse promoters by restriction PCR, we found that, like zebrafish MG, mouse promoter methylation levels of pluripotency factors and genes associated with regeneration in zebrafish had low methylation levels (Fig. 5*I* and Fig. S7*B*). This suggests that, like

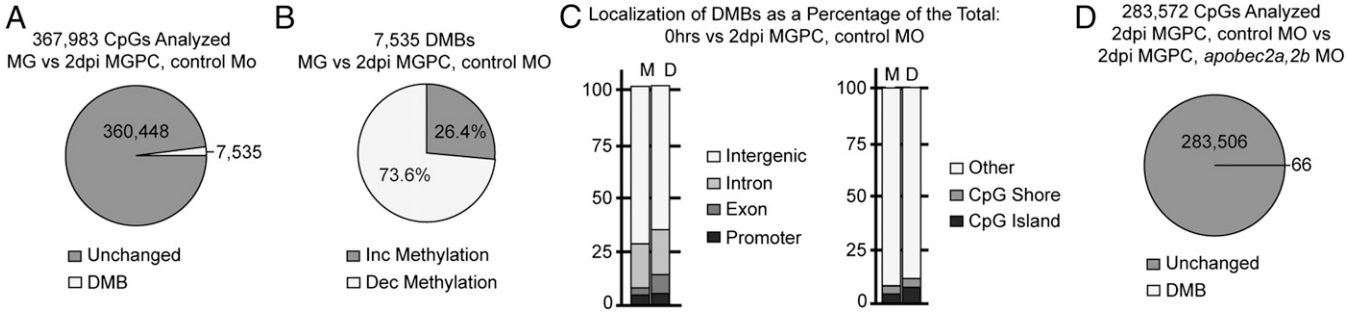

Fig. 4. Site-specific, active DNA demethylation predominates at 2 dpi and is independent of Apobec2a,2b. (*A–C*) RRBS comparison of quiescent MG (*gfap:gfp*) and 2-dpi MGPCs treated with control MOs. Quantification (*A*), classification (*B*), and localization (*C*) of DMBs as performed in Fig. 2. DNA methylation is regulated by 2 dpi (incipience of MG proliferative response), suggesting that these changes are actively made (9). Most DMBs are localized to intergenic, CpG-poor regions. (*D*) RRBS comparison of 2-dpi MGPCs treated with control or *apobec2a,2b* MOs indicates that the knockdown of Apobec2a,2b does not impact site-specific DNA demethylation.

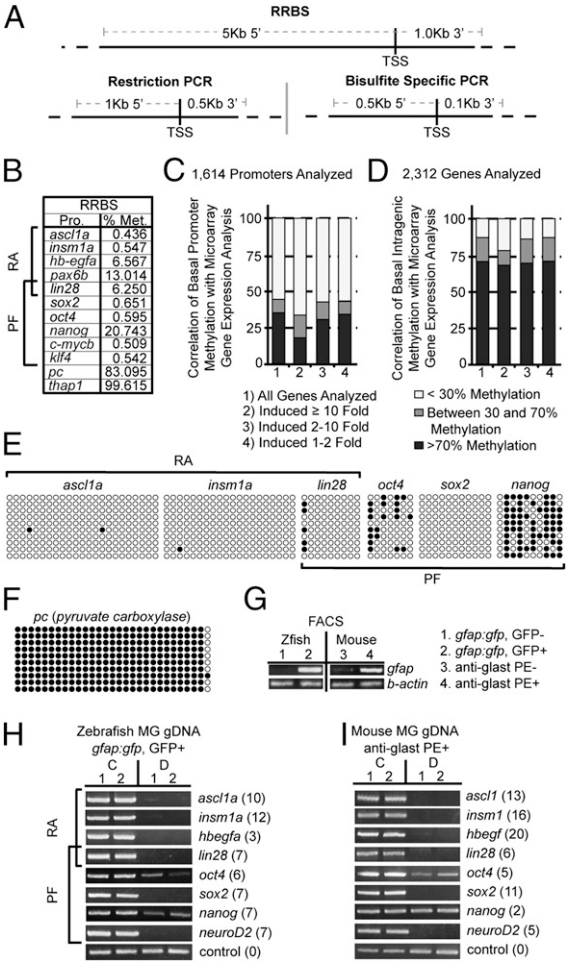

Fig. 5. MG basal DNA methylation profile suggests that they are to an extent poised for a rapid regenerative response. (*A*) Graphic depicting target regions analyzed by RRBS, bisulfite PCR, and restriction PCR. (*B*) Average promoter methylation levels calculated using quiescent MG RRBS data indicate that many pluripotency factor (PF) and regeneration-associated (RA) genes have low basal methylation levels. (*C* and *D*) Correlation of basal promoter methylation levels with microarray gene expression data indicates that highly induced genes have lower than average promoter (*C*) and intragenic (*D*) methylation levels. (*E* and *F*) Bisulfite PCR analysis of promoter regions characterized as having low (*E*) or high (*F*) basal methylation levels by RRBS. Each horizontal line of circles represents an independent clone. Filled and unfilled circles represent methylated and unmethylated cytosines, respectively. (*G*) Validation of MG enrichment following FACS of zebrafish *gfap:gfp* transgenic fish retinas (GFP-negative and GFP-positive populations) and mouse retinas (anti-GLAST PE-negative and anti-GLAST PE-positive populations) using RT-PCR. (*H*) Zebrafish promoter restriction PCR following genomic digest with HpaII, HpyCH4IV, and BstuI (D) or mock/control, undigested (C) using MG (*gfap:gfp*, GFP-positive) gDNA. The number of potential restriction cut sites is indicated next to each target name. (*I*) Restriction PCR was carried out as in *H* using mouse MG (anti-GLAST PE-positive) gDNA. PF and RA gene promoters of both zebrafish and mouse MG appear to be lowly methylated.

zebrafish MG, mouse MG may be somewhat poised for a regenerative response if provided the proper stimulation.

Discussion

During zebrafish retina regeneration, MG transition from a quiescent supportive cell to an MGPC. This transition is accompanied by the increased expression of pluripotency and other regeneration-associated genes (14–17, 19–22). Previously studied somatic cell reprogramming events found strong correlations between the increased expression of pluripotency factors and decreases in their promoter methylation levels (7, 27, 28). Likewise, we hypothesized that the regulation of DNA methylation would accompany the reprogramming of MG to MGPCs in the injured retina. Indeed, we found that as MG transition to an MGPC, their genome undergoes dynamic changes in DNA methylation. This analysis of the MG DNA methylation landscape as it transitions from a differentiated support cell to an MGPC provides unique insight into the reprogramming process as it takes place in an intact animal.

When we began these studies, we suspected that MG reprogramming may share characteristics with somatic cell reprogramming based on their common need to reawaken gene expression programs silenced during development. Quite surprisingly, and unlike somatic cell reprogramming, we found pluripotency factor gene promoter methylation levels do not change during MG reprogramming, but rather are already locked into a hypomethylated state. Similarly, we found that genes that are highly induced and necessary for MG reprogramming harbor hypomethylated promoters that remain unchanged throughout the reprogramming event. These observations suggest that MG are (to an extent) preprogrammed for a regenerative response and may be more stem cell-like than previously thought (29). This type of cellular preprogramming has also been noted during zebrafish development (24). The basal hypomethylation of MG may aid the regenerative process by increasing the speed and efficiency of MGPC formation, both of which are higher than those seen during iPSC generation. Interestingly, blocking DNA methylation improves the efficiency of iPSC generation (4).

With these thoughts in mind, we investigated the methylation status of a subset of these promoters in mice to ascertain whether DNA methylation at these locations could account (at least partially) for the limited regenerative potential of mouse MG. Surprisingly, these mouse promoters also exhibited low methylation levels similar to their zebrafish counterparts. Thus, understanding why they remain repressed and devising ways to neutralize this repressive environment may facilitate mammalian MG reprogramming. One candidate for this repression, *let-7*, appears to inhibit regeneration in both zebrafish and *Caenorhabditis elegans* (14, 30). Interestingly, Ascl1a inhibits *let-7* expression during zebrafish MG reprogramming (14), and its overexpression in postnatal mouse MG enhanced their reprogramming (31). We suspect that this latter effect is mediated by inhibition of *let-7* and hypothesize that *let-7* is a key factor contributing to the limited regenerative potential of mammalian MG.

Although we did not find methylation changes occurring in the promoters of pluripotency factor and early-induced regeneration-associated genes, we did identify a changing DNA methylation landscape as MG reprogram to MGPCs. During MGPC formation (0–2 dpi), DNA demethylation predominates and may underlie some of the gene expression changes noted during this time. Indeed, many promoter demethylation events are associated with increased gene expression. However, other mechanisms must also be at play because some of the best-studied regeneration-associated genes do not undergo changes in DNA methylation. These genes may be directly regulated by other events such as histone modifications and availability of transcription factors. Thus, changes in DNA methylation may be necessary for MG reprogramming, but may not be sufficient.

The predominance of DNA demethylation as MG transition to an MGPC is intriguing, and we suspected that Apobec2 proteins were involved in this process based on previous studies showing that their knockdown inhibits retina regeneration (22) and that they contribute to DNA demethylation in zebrafish embryos and adult mouse brains (28, 32). Surprisingly, our studies suggest that they do not have a significant impact on site-specific active DNA demethylation during MG reprogramming. Thus, other genes potentially involved in DNA demethylation, such as Gadd45 (28, 33, 34) and Tet proteins (35), may be better targets for investigating the impact of DNA demethylation during MG reprogramming. The role that Apobec2 proteins play during MG

reprogramming remains to be determined. It is possible that these proteins, like other Apobecs, catalytically edit RNAs, but it is also possible that they perform some function that is independent of a deaminase activity. Indeed, some have questioned whether Apobec2 proteins are catalytically active and whether they bind polynucleotides (36–39). Further work is needed to shed light on the mechanism of these proteins.

From 2 to 4 dpi, when MGPCs are expanding, the DNA methylation landscape shifts from one that is predominately driven by demethylation (2 dpi) to one with increasing levels of de novo methylation (4 dpi). We note that at this time, MGPCs are at the peak of their proliferative phase and may be preparing for differentiation. Perhaps the increased methylation noted at 4 dpi contributes to the differentiation of MGPCs as they begin to repair a damaged retina. Consistent with this idea, pharmacological inhibition of this methylation was detrimental to proper lamination and differentiation of these MGPCs.

In summary, our comprehensive analysis of the DNA methylation landscape as MG transition to an MGPC and generate progenitors for retinal repair provides a unique view of MG reprogramming as well as an important resource for understanding the function of DNA methylation in cellular reprogramming and retina regeneration.

Materials and Methods

Animals, Retinal Injury, MO Delivery, and FACS. Transgenic fish and retinal lesions were described (9, 40). MO delivery was facilitated by electroporation (22). Zebrafish target cell populations were purified by FACS (17). Mouse MG and non-MG were isolated by FACS of dissociated retinas (Neural Tissue Dissociation Kit; Miltenyi Biotec) stained with anti-GLAST PE (ACSA-1) (Miltenyi Biotec). See *SI Materials and Methods*.

RNA Isolation, gDNA Isolation, and PCR. Total RNA was isolated using TRIzol (Invitrogen) and was DNase-treated (Invitrogen). cDNA synthesis was performed by using random hexamers and SuperScript II (Invitrogen). Primers are listed in Table S2. gDNA was used for bisulfite and restriction PCR following isolation using the PureLink Genomic DNA Mini Kit (Invitrogen). Bisulfite PCR was performed on bisulfite-converted gDNA (EZ DNA Methylation Direct Kit; Zymo) (7, 28). See *SI Materials and Methods*.

Immunofluorescence. Immunofluorescence was as described (22). *See SI Materials and Methods*.

RRBS. Libraries were prepared as previously described (41) using components of the Illumina TruSeq DNA Sample Preparation Kit. Sequencing was performed on an Illumina HiSeq 2000. Sequence data were analyzed as published (41, 42). Sequencing data are available in the Gene Expression Omnibus database (accession no. GSE50717). Detailed information is provided in *SI Materials and Methods*.

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