**High Mobility Group Box-1 (HMGB1; Amphoterin) Is Required for Zebrafish Brain Development**

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_Hmgb1_ (high mobility group box-1; amphoterin) is highly expressed in brain during early development of vertebrate and nonvertebrate species. However, its role in brain development remains elusive. Here we have cloned the zebrafish _Hmgb1_ and specifically manipulated _Hmgb1_ expression using injection of morpholino antisense oligonucleotides or _Hmgb1_ cRNA. The HMGB1 knockdown morphants produced by injection of three different morpholino oligonucleotides display a characteristic phenotype with smaller size, smaller brain width, and shorter distance between the eyes. Closer examination of the phenotype reveals severe defects in the development of the forebrain that largely lacks catecholaminergic neural networks. The HMGB1 morphant is deficient in survival and proliferation of neural progenitors and displays fewer cell groups expressing the transcription factor Pax6a in the forebrain and aberrant Wnt8 signaling. The mechanism of HMGB1-dependent progenitor survival involves the neuronal transmembrane protein AMIGO (amphoterin-induced gene and orf), the expression of which is regulated by HMGB1 in vivo. Our data demonstrate that HMGB1 is a critical factor for brain development, enabling survival and proliferation of neural progenitors that will form the forebrain structures.

The high mobility group box-1 protein (HMGB1; also designated as HMG1 and amphoterin) is an abundantly occurring parental form of the HMG proteins (for review, see Ref. 1). HMGB1 is an exceptional member in the family of HMG-box proteins; depending on the cell type and its activation state, HMGB1 displays a nonnuclear localization and is secreted from cells, in contrast to most HMG-box proteins that are strictly bound to the cell nuclei (for recent reviews, see Refs. 2 and 3). During the last few years, the extensive literature dealing with HMGB1 functions has mainly focused on extracellular regulation of cells by HMGB1. HMGB1 can be passively released from injured cells, but it is also actively secreted due to several types of stimuli such as cell contact with extracellular matrix and cytokine stimulation of cells (2). Acetylation of lysine residues of HMGB1 has been shown to act as a signal leading to extracellular export via a non-classical secretory pathway (4). HMGB1 functions are currently mainly associated with binding to the cell surface receptor RAGE (receptor for advanced glycation end products), but Toll-like receptors have been increasingly suggested as membrane receptors of HMGB1 (for review, see Ref. 2).

Compared with the extensive recent literature dealing with the pathophysiological roles of HMGB1 in inflammation, much less attention has been paid to its physiological roles. The _Hmgb1_ knock-out mouse survives until early postnatal age, and problems in glucose homeostasis have been suggested to cause multiorgan failure in these mice (5).

HMGB1 was isolated from developing rat brain using neurite outgrowth in embryonic forebrain neurons as a readout in protein fractionation (6). These studies provided the initial evidence of HMGB1 as an extracellularly acting protein (6–8), which has become the major line of HMGB1 research during the last few years. HMGB1 was found to bind strongly to heparin/heparan sulfate and to be highly expressed in embryonic rat brain (6). RAGE acts as a receptor in HMGB1-induced extension of neurites (for review, see Ref. 2). Furthermore, exogenously added HMGB1 has been shown to enhance survival of neuronal cells in a RAGE-dependent manner (9). In cancer cells, HMGB1 has been shown to bind to RAGE and to enhance tumor growth and spread (10). Despite these in vitro and in vivo findings, which would be compatible with a developmental role for HMGB1, its possible role in brain development has not been explored.

The forebrain is the part of the nervous system that has undergone the most dramatic changes during vertebrate evolution. The early organization of the forebrain subdomains is conserved in all vertebrates. To understand the origins of the vertebrate forebrain, comparisons of gene expression patterns have been recently carried out in several nonvertebrate and early vertebrate organisms (see for example Refs. 11–13) representing species at the dawn of the vertebrate brain development. _Hmgb1_ is highly expressed in early embryonic brain and might, therefore, have a role in the development of ancestral forms of complex brain structures.

The findings explained above, neurite outgrowth-promoting and survival-enhancing effects on cultured neuronal cells and high expression in early brain structures, raise the question of whether _Hmgb1_ would be one of the genes required for brain development. In the current study we have addressed this question using zebrafish as a vertebrate model in which develop-
ment of different nervous system structures can be easily followed. HMGB1 knockdown experiments in vivo using morpholino oligonucleotides clearly demonstrate defects in brain development, in particular in anterior neural structures. We suggest that in developing forebrain, HMGB1 is required for maintenance of proliferating cells/stem cells that give rise to neurons.

**EXPERIMENTAL PROCEDURES**

**Experimental Animals**—An outbred zebrafish (*Danio rerio*) strain from a local resource, Turku line, was used in this study for its steady yield of embryos (14, 15). Fish feeding, breeding, and maintenance were done according to Westerfield (16). The experiment permits were obtained from the University of Helsinki Committee for animal experiments and the Office of the Regional Government of Southern Finland, in agreement with the ethical guidelines of the European convention. We express the embryonic ages in hours post-fertilization (hpf) and days post-fertilization (dpf). To prevent pigment formation, 0.2 mM 1-phenyl-2-thiourea (Sigma) was added to the media of embryos to be studied before 3 dpf shortly after spawning.

**RT-PCR and Quantitative RT-PCR**—Total RNA from 2 dpf larvae was extracted with NucleoSpin RNA XS kit. RNA was reverse-transcribed in a reaction containing 1 µg of RNA, 0.25 mM dNTP-mix, 1 mM random nonamers, 20 units of recombinant RNasin® (Promega), 200 units of Moloney murine leukemia virus (MMLV)-RT (Promega) in 1× MMLV reaction buffer. 2 µl of the reverse transcription mixture was then used for polymerase chain reaction with gene-specific primers. The primers 5′-ACA TCC ACA TAC AGC CAT TGC-3′ and 5′-GGG AAG AGG GAT GTG GTT GGA-3′ were used for cloning the 963-bp zebrafish HMGB1 full-length transcripts of antisense strand. The PCR cycling parameter was set at 95 °C for 5 min, denaturation at 95 °C for 29 cycles, multiple rounds of 30 s at 95 °C for 30 s, annealing/detected at 62 °C for 30 s, back to denaturation for 39 cycles, hold at 4 °C.

**Preparation of Tissues**—Zebrafish embryos were collected and transferred onto a Petri dish with E3 medium (miniQ water containing 5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, and 0.33 mM MgSO₄). The fish were killed by keeping them on ice for more than 10 min following by decapitation or fixation. Larval fish were fixed whole with 2% paraformaldehyde in 0.1 M phos-
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phosphate buffer at room temperature for 2 h or at 4 °C overnight, and brains of 5-dpf larvae were dissected under a stereomicroscope after the fixation (15). The fixed samples could be saved in phosphate buffer for up to 2 weeks.

Whole-mount in Situ Hybridization—Whole-mount in situ hybridization was carried out as described previously (17) using the specific probes (18) of Pas2a (the ZIRC cb378), Pas6a (ZIRC cb280), and Krox20 (ZIRC cb427). Larvae at 30-hpf (Prim-5) the specific probes (18) of Pas2a (the ZIRC cb378), Pas6a (ZIRC cb280), and Krox20 (ZIRC cb427). Larvae at 30-hpf (Prim-5)

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spondingly) was injected into the yolk of a 1–4-cell embryo that was allowed to develop at 28.5 °C. For cRNA rescue experiments, 0.1 μg/μl cRNA was mixed with MO injection solution after the heating. All injections were done with an injector (WPI PV830 Pneumatic Pico Pump) and a micromanipulator (Narishige, Tokyo, Japan).

Cell Death and Proliferation Detection—Whole-mount staining of 28-hpf larval apoptosis was performed using the DeadEnd™ Fluorometric TUNEL system (Promega (23)), and whole-mount staining of 80-hpf larval cell proliferation was studied with Click-iT Edu Alexa® Fluor 555 imaging kit (Invitrogen) using the protocol recommended by the manufacturer. Unlike assays using bromodeoxyuridine, Click-iT® EdU assays are not antibody-based and, therefore, do not require DNA denaturation for detection of the incorporated nucleoside. Instead, Click-iT® EdU utilizes click chemistry for detection in a variety of Alexa Fluor® dye fluorescent readouts. (Invitrogen, #C10338).

Microscopy—Confocal imaging of 5-dpf brains stained with anti-TH antibodies was carried out as described (24). Zeiss LSM5 Pascal confocal microscopy system with air (10× dry) and water immersion objectives (high magnification, 25 or 63×) was used for imaging of 28-hpf larvae stained with anti-AMIGO1 and anti-HMGB1 antibodies. The fluorophores were excited with the 488- or 568-nm lines from an argon-krypton laser (Omnichrome; Melles Griot, Carlsbad, CA). Cross-talk between the channels and background noise was eliminated with sequential scanning and frame averaging as described earlier (15). Stacks of images taken at 1–1.2-μm intervals were compiled to make maximum intensity projection images. For each scan, uninjected larval samples were scanned first for imaging normalization. All morphants were then scanned using the same experimental details. Specimens for light microscopy were examined with inverted light microscopy using Olympus IX 70 connected through a CCD camera to the Analysis® software. High resolution whole-mount in situ images were obtained with a digital MicroFire S99808 camera (Optronics) attached to an Olympus BX51 epifluorescence microscope (Olympus, Tokyo, Japan).

The acquired images were further processed with Adobe Photoshop 8.0 software (Adobe Systems, San Jose, CA). Anatomical structures of larval brain were named and numbered using the neuroanatomical atlas of adult zebrafish brain (25, 26) and the atlas based on location of TH neurons in 5-dpf larval fish (14, 24).

Analytical Software—All statistical analyses were carried out using OriginPro 7.5. Intensities of the Western blot bands were examined with QuantityOne 4.6.2 (Bio-Rad) software. HMGB1 protein sequence alignments of different species (Ensembl) and statistical analysis were carried out using Geneious pro trial 4.8.2 ( Biomatters Ltd.).

RESULTS

Zebrafish Hmgb1—The zebrafish Hmgb1 gene has the same exon-intron organization (supplemental Fig. S2) as the mammalian Hmgb1. The full-length zebrafish Hmgb1 DNA was cloned by using mRNA from zebrafish larvae and primers designed according to the putative homologous sequence found in the Zv8 data base (Ensembl, search Danio rerio). Blast searches identified Hmgb1 as the closest homologue within the mammalian sequences. Comparisons of the deduced amino acid sequence (supplemental Fig. S1) to the human or mouse HMGB1 and HMGB2 sequences displayed 86 and 78% similarity, respectively. As in mammals, the zebrafish sequence corresponds to the protein having two homologous HMG boxes (HMG boxes A and B) followed by the acidic tail consisting of only glutamate and aspartate residues (Fig. 1A). The deduced amino acid sequence is slightly shorter (205 amino acids) than the mammalian one (214 amino acids).

Detection of Zebrafish HMGB1 Using Specific Antibodies—We have previously produced a series of affinity-purified anti-protein and anti-synthetic peptide antibodies that detect the mammalian HMGB1 (19). Antibodies against a synthetic peptide (anti-peptide IV; see Ref. 19) corresponding to a highly conserved area immediately before and at the beginning of the acidic tail (Fig. 1A). The deduced amino acid sequence of the protein having two homologous HMG boxes (HMGB1 and BMGB2) displayed 86 and 78% similarity, respectively. As in mammals, the zebrafish sequence corresponds to the protein having two homologous HMG boxes (HMG boxes A and B) followed by the acidic tail consisting of only glutamate and aspartate residues (Fig. 1A). The deduced amino acid sequence is slightly shorter (205 amino acids) than the mammalian one (214 amino acids).

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pholino antisense experiments specifically targeting HMGB1 (see e.g. the Western blots in Fig. 2).

Inhibition of HMGB1 Expression in Zebrafish Larvae with Morpholino Oligonucleotides—Gene-specific antisense MOs have been widely used to inhibit gene expression in zebrafish larvae (27). We designed three MOs (see “Experimental Procedures” and supplemental Fig. S2) expected to interfere in Hmgb1 expression. The first one (MO1) targets a sequence that includes the ATG site (from 11002 to 11001 compared with the ATG site) and is expected to inhibit HMGB1 translation. The other two oligonucleotides (MO2 and MO3) target splice sites in the Hmgb1 pre-mRNA. MO2 is expected to cause an exon deletion, whereas MO3 is expected to cause an intron insertion.

Noninjected larvae and larvae injected with the five mispaired FIGURE 2. Dosages of HMGB1 MOs required for specific knockdown of HMGB1 expression and rescue of the expression by coinjecting Hmgb1 cRNA. A, shown is Western blotting of HMGB1 from lysates of 3-dpf larvae that had been injected with different dosages of the MOs. MO1 causes a more prominent inhibition of HMGB1 expression than MO2 or MO3. B, shown is quantification of the Western blotting bands in A by plot density analyses. MO1 (2 ng) causes >80% knockdown of HMGB1 expression, and MO2 and MO3 (4 ng each) cause >50% inhibition of expression. C, statistics of the death rate in different MO-injected 24-hpf larvae are shown. By increasing the MO1 injection dosage from 1 to 4 ng, the death rate of the MO1-injected morphants displays a significant increase (compared by one-way ANOVA followed by Fisher’s LSD post hoc test and Bonferroni correction; *, p < 0.001), from less than 8% to around 20%. At the same doses, MO2 or MO3 do not cause an appreciable change. For each group, 300 larvae were used in 6 independent injections; 50 larvae were injected in each experiment. D, shown is RT-PCR of 2-dpf larval total RNA using primers from both ends of the coding sequence of the Hmgb1 cDNA (615 bp). MO1 does not cause an appreciable change in the analysis, whereas a band of 440 bp is found in the MO2-injected larval sample corresponding to exon 3 deletion. In the MO3-injected larval sample, the 615-bp Hmgb1 PCR product is clearly reduced compared with the controls. GADPH was used as the template control. E and F, inhibition of HMGB1 expression by the MOs and rescue of the expression by coinjection of the Hmgb1 cRNA are shown. The three groups of larvae injected with the morpholino oligonucleotides (2 ng of MO1; 4 ng of MO2 or MO3) display a clearly reduced expression of the HMGB1 protein compared with the uninjected group or the 5 mis MO-injected group. A Western blot is shown in E, and quantification of the Western blotting band intensities from four replicates is shown in F. In the groups of larvae injected with MO1, MO2, or MO3, the Hmgb1 expression is significantly reduced compared with the MO/cRNA-coinjected larvae (compared by one-way ANOVA followed by Fisher’s LSD post hoc test and Bonferroni correction; *, p < 0.01). In the group with the best rescue effect (MO2/cRNA), the Hmgb1 expression is ~80% of uninjected controls. In the group with the lowest rescue effect (MO1/cRNA), the expression is >60% of uninjected controls. For Western blotting, 20 larvae of each injection group were selected randomly for sample preparation. The experiment was repeated three times by independent injections. The error bars in (C and F) indicate the S.E. values.
MOs (5mis MO; having five non-pairing nucleotides compared with MO1) were used as controls.

In larval lysates, HMGB1 was specifically detected as the protein with a 28-kDa molecular mass (Fig. 2A). We used Western blotting of 3-dpf larval lysates to evaluate the effects of the MOs (at the doses 1–6 ng) on HMGB1 protein expression. Estimated from the Western blots (Fig. 2, A and B), 2 ng of MO1 were required to cause more than 50% inhibition of the protein expression, whereas somewhat higher doses of MO2 and MO3 (4 ng) were required for a similar inhibition. We selected these doses for further experiments aiming at studies on organ development as they allow early development and high proportions of viable larvae (Fig. 2C).

RT-PCR analysis (Fig. 2D) of the total RNA extracted from 2-dpf MO1-injected larvae revealed expression of Hmgb1 mRNA at the same level as in controls, as expected. In contrast, in MO2-injected larvae the normally occurring mRNA was strongly reduced, and a band corresponding to exon 3 deletion was found. In MO3-injected larvae, expression of the normally occurring mRNA was strongly reduced.

The MOs were designed so that they may not inhibit the capped Hmgb1 cRNA injected in rescue experiments together with the MOs. MO1 extends from −15 until +10 compared with the ATG site and MO2 and MO3 target splice sites of the pre-mRNAs (supplemental Fig. S2). Western blotting showed that in the selected doses (Fig. 2E; quantification from replicate filters in Fig. 2F) MO1 causes 80–90% inhibition in the protein expression, whereas MO2 and MO3 cause 70 and 50–60% inhibition correspondingly. Coinjection of the Hmg1 mRNA together with MO1, MO2, or MO3 caused a significant rescue of the protein expression (Fig. 2, E and F).

Expression of HMGB1 during Early Development of Zebrafish—Expression of Hmgb1 mRNA has been mapped at different stages of zebrafish development (see The Zebrafish Model Organism Database). Hmgb1 is expressed in blastula, gastrula, and segmentation stages ubiquitously until 14 hpf. After this, HMGB1 is mainly expressed in brain and other parts of the nervous system until 5 dpf, when the expression is down-regulated. Ventral mesoderm also expresses Hmgb1 during organogenesis.

To gain further insight into HMGB1 expression in zebrafish embryos, we carried out whole-mount immunostaining experiments of 28-hpf larvae (Fig. 3) using antibodies against HMGB1 (anti-peptide IV; see above). The 28-hpf larvae have already completed primary neurogenesis and are entering into the stage of secondary neurogenesis (28). HMGB1 was found to be prominently expressed in forebrain, in particular in rostral telencephalon and telencephalon close to the ventricular wall, in pretectum, and at the anterior part of the diencephalic ventricular wall (Fig. 3 and supplemental Figs. S3–S5). Immunostaining on these regions is specific as it is strongly reduced or disappears in MO1 injected larvae. Furthermore, the coinjected Hmgb1 cRNA rescued immunostaining, although the immunostaining pattern is different from that caused by the endogenous HMGB1 expression (Fig. 3 and supplemental Figs. S3 and S4). The areas where HMGB1 is expressed are populated by brain stem cells/neuronal progenitor cells (29) during the primary and secondary neurogenesis (28). In addition to the fore-
brain, HMGB1 was found to be expressed symmetrically along the brain midline in the spinal cord and in the notochord (supplemental Fig. S6).

AMIGO1 (amphoterin-induced gene and orf) is a nervous system specific transmembrane protein whose expression is induced in neuronal cells by extracellular matrix-associated HMGB1 (20). We used AMIGO1 as a neuronal plasma membrane marker in double-immunostaining with HMGB1. Immunostaining using the anti-HMGB1 and anti-AMIGO1 ectodomain antibodies revealed partial colocalization at the plasma membrane (Fig. 3 and supplemental Figs. S3 and S4). A high resolution view of HMGB1/AMIGO1 double-immunostaining in diencephalon revealed a patchy detection of both proteins that partially colocalize at the plasma membrane (Fig. 3 and supplemental Fig. S5). Visualization of membrane structures with the lipid-soluble dye DiI revealed a similar localization at the membrane level (not shown).

Furthermore, immunostaining experiments showed that MO1 injection down-regulates both the HMGB1 and AMIGO1 expression, whereas expressions of both proteins are up-regulated by coinjection of the Hmgb1 cRNA (Fig. 3 and supplemental Figs. S3 and S4). The expression and function of AMIGO1 may, therefore, be linked to those of HMGB1 during forebrain development.

Morphological Characteristics of the HMGB1 Knockdown Morphants—Each MO caused phenotypic changes at the dose range of 1–6 ng per embryo. To analyze the phenotypic effects of the MOs, we selected the lowest doses causing a 50% or higher inhibition of HMGB1 protein expression as evidenced by Western blotting analysis but not compromising the viability of the embryos (see above and Fig. 2).

Examination of gross morphology of MO-injected zebrafish revealed prominent changes starting at the early stages of development (1–2 dpf). The morphant phenotype can be easily recognized based on smaller size, curling tails, smaller brain width, and shorter distance between the eyes (Fig. 4A and supplemental Fig. S7). They are immobile and stay alive until about 1 week. All three MOs (MO1, MO2, and MO3) produced a very similar change, but there was a difference in the frequency of the characteristic phenotype observed among the three MOs. Analysis of 3–dpf larvae revealed that injection of MO1 (2 ng) caused the characteristic phenotype in 80–90% of the injected zebrafish with somewhat lower frequencies for MO2 and MO3 injections (4 ng of each MO; Fig. 4B). This finding agrees with the Western blot analysis showing that MO1 causes a more prominent and effective down-regulation of HMGB1 expression compared with MO2 or MO3 injections (4 ng of each MO; Fig. 4B). This finding agrees with the Western blot analysis showing that MO1 causes a more prominent and effective down-regulation of HMGB1 expression compared with MO2 or MO3 injections (4 ng of each MO; Fig. 4B).

Immunostaining of TH1-positive networks revealed prominent changes starting at the early stages of development (30 hpf) displayed a disordered pat-
the midline. The phenotype was essentially rescued in MO1/\textit{Hmgbl} cRNA-coinjected larvae, with only slight changes compared with wild-type controls (Fig. 4C).

\textbf{Cell Survival and Proliferation in the HMGB1 Knockdown Zebrasfish}—Previous studies have shown that HMGB1 enhances survival of cultured embryonic cells (9). Because HMGB1 is robustly expressed close to ventricular walls populated by stem cells/proliferating cells, we reasoned that HMGB1 might be required for survival and proliferation in early forebrain. TUNEL staining revealed abundantly apoptotic cells in forebrain and hindbrain in 28-hpf MO1-injected zebrafish larvae (Fig. 5A). At this stage, apoptosis has been shown to be very rare in the normal developing brain (32), in agreement with our analysis of the wild-type larvae used as controls (Fig. 5A). Coinjection of \textit{Hmgbl} cRNA was essentially able to reverse the effect (Fig. 5A), suggesting that apoptosis was specifically caused by inhibition of HMGB1 expression.

Based on TUNEL labeling, the HMGB1 knockdown larvae display apoptosis both in forebrain and hindbrain, which is somewhat unexpected as the morphological changes were largely restricted to the forebrain compared with other parts of the nervous system (see above). Yet the cRNA rescue experiments clearly argued for a specific effect (Fig. 5A). As an additional set of control experiments, we therefore studied the possible p53 activation that is suggested as a control of off-target effects in siRNA and MO knockdown technologies (21). We did not find any evidence of p53 activation that would explain apoptosis or other effects in the knockdown experiments using any of the three HMGB1 MOs (supplemental Fig. S8, \textit{A} and \textit{B}). Furthermore, inclusion of p53 MO is recommended to reduce the risk of off-target effects in knockdown experiments, but this approach did not change the characteristic larval phenotype of the HMGB1 knockdown (supplemental Fig. S8C) or the numbers of TUNEL-positive larvae (not shown).

If HMGB1 is required to maintain proliferating cells/stem cells in developing brain, one would expect to see a difference in DNA synthesis in brain regions populated by such cells in experiments where the HMGB1 expression level is manipulated. EdU labeling revealed prominent staining that was strongly reduced in the brain of MO1-injected larvae (shown for 3-dpf larvae in Fig. 5B). The staining suggesting cell proliferation was rescued by injecting \textit{Hmgbl} cRNA together with MO1. Interestingly, EdU labeling was somewhat more widespread along the midline close to the brain ventricles in forebrain and midbrain compared with the controls without cRNA injection (Fig. 5B). Correspondingly, \textit{Hmgbl} cRNA-injected larvae also showed diffuse staining with the HMGB1 antibody (Fig. 3). This is to be expected as the injected coding sequence lacks regulatory elements and may, therefore, cause ubiquitous HMGB1 expression. HMGB1 thus appears to affect cell proliferation on regions where it is not even endogenously expressed (Fig. 3).
by Hmgb1 cRNA coinjection in most of the embryos studied (see Fig. 6 for morphology and the percentages of embryos lacking Pax6-positive cells in telencephalon). In contrast to the telencephalic area, Pax6a expression appeared normal in more posterior areas of the central nervous system. We did not detect any major differences in the expression patterns of Pax2a or Krox20 (Fig. 6). These results are in agreement with the morphological findings (see above), suggesting that forebrain development is especially vulnerable to down-regulation of HMGB1 expression.

Wnt Signaling in the HMGB1 Knockdown Zebrafish—Wnt signaling plays a key role in the development of vertebrates and nonvertebrates (for review, see Ref 37). In particular, Wnt8 signaling has been connected to forebrain development and is
known to become dysregulated in Pax6\(^{-/-}\) embryos (34, 38). We, therefore, decided to study whether HMGB1 knockdown affects Wnt8 signaling. Surprisingly, Wnt8a\(^1\) mRNA was up-regulated 4–5-fold in MO1-injected larvae, and the effect was rescued by injection of the Hmgb1 cRNA. In addition, Wnt8a\(^2\) and Wnt8b mRNAs were up-regulated about 2-fold and 2–3-fold, respectively (Fig. 7A).

A key question is, therefore, whether the changes in mRNA levels reflect on protein levels and cell signaling. Because the Wnt proteins are hard to detect, we chose to take an indirect approach by following expression of the β-catenin protein that is known to become stabilized and, therefore, up-regulated during canonical Wnt signaling. Western blotting showed that the β-catenin level is indeed specifically up-regulated about 3-fold in the HMGB1 morphants (shown for 3-dpf morphants in Fig. 8, A and C). As expected, a corresponding rescue effect was observed in EdU staining of TH1-expressing neuronal networks to evaluate the possible rescue effect. In more than 60% of larvae, TH1-positive networks became detectable in telencephalon when AMIGO1 cRNA was coinjected with MO1 (Fig. 8, C and D). The corresponding value for the MO1/Hmgb1 cRNA-coinjected groups was about the same, whereas GFP cRNA coinjected control did not cause a significant rescue effect (Fig. 8D).

Furthermore, rescue experiments suggested that AMIGO1 is involved in the survival and proliferation-enhancing mechanism of HMGB1. Abundant TUNEL staining was found in a high proportion of MO1-injected larvae compared with mis MO-injected or un.injected larvae; injection of Hmgb1 cRNA or AMIGO1 cRNA both caused a clear rescue effect (see the qualitative comparison in Fig. 9A and a comparison from replicate experiments in Fig. 9C). Coinjection of p53 MO or GFP cRNA did not change the numbers of TUNEL-positive larva (not shown). As expected, a corresponding rescue effect was observed in EdU staining of proliferating cells (Fig. 9, B and C).

**DISCUSSION**

Previous \textit{in situ} hybridization studies have shown that during organogenesis,HMGB1 mRNA accumulates in early brain structures in many nonvertebrate species, such as amphioxus (12) and \textit{Xenopus} (13) and the basal vertebrate lamprey (11). In vertebrates, a high expression level of HMGB1 (amphoterin) has been demonstrated in embryonic rat brain compared with the adult brain (6), and mapping studies using \textit{in situ} hybridization have shown that during organogenesis in zebrafish HMGB1 is essentially a nervous system protein that is abundantly expressed in brain (see The Zebrafish Model Organism Database). However, excluding \textit{in vitro} studies using primary...
forebrain cells and neuroblastoma cells (6, 8), the possible role of HMGB1 in brain development remains to be explored.

**HMGB1 Regulates Forebrain Development**—The current study shows that HMGB1 is essential for forebrain development in zebrafish. Several lines of experiments argue for specificity in our knockdown experiments. Western blotting shows that the HMGB1 protein is down-regulated by three different HMGB1 antisense MOs, and the phenotypic changes parallel the HMGB1 down-regulation. Furthermore, similar morphants were observed for all three antisense MOs but not for the control MO (5mis MO). Finally, clear rescue effects can be observed when the Hmgb1 cRNA is injected in knockdown experiments.

Because HMGB1 is highly expressed in the central nervous system during early development in all species studied so far, we suggest that the role in brain development is a conserved phenomenon in evolution. In particular, the diencephalic and telencephalic forebrain structures appear vulnerable to the down-regulation of HMGB1 expression, and we suggest that this highly conserved gene has an important function enabling survival and proliferation of stem cells/precursor cells that will form the forebrain structures. It is still unclear to us why the forebrain appears especially vulnerable to the down-regulation of HMGB1 expression. A possible reason to this is that, compared with other parts of the brain, high numbers of cells have to be produced and added to the complex network structures in the developing forebrain. Therefore, the forebrain would be more critically dependent on survival/proliferation enhancing factors than lower parts of the nervous system, leading to the situation that down-regulation of only one factor causes massive perturbation of forebrain structures.

**HMGB1 as an Extracellular and Intracellular Factor Regulating Forebrain Development**—Proliferation and differentiation of neural progenitors is heavily dependent on the specialized microenvironment, the niche in which the cells reside. The cells are regulated within these niches by soluble and membrane-bound molecules and by extracellular matrix. One should, therefore, consider whether HMGB1 participates in

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**FIGURE 8.** Inhibition of AMIGO1 expression in the HMGB1 knockdown morphants and the rescue effect of the AMIGO1 and Hmgb1 cRNA on the development of catecholaminergic neural networks in the HMGB1 knockdown morphants. A, Western blotting of AMIGO1 and of Hmgb1 using β-actin as the control for each protein is shown. B, quantification of the Western blotting result shown in A is based on four independent injections; 20 larvae of each injection group were randomly selected for sample preparation. The AMIGO1 and HMGB1 expression level of uninjected larvae has been set as 100%. Expression of both HMGB1 and AMIGO1 is strongly inhibited by MO1, whereas the 5mis MO does not cause a significant inhibition. The HMGB1 cRNA rescues the expression of both HMGB1 and of AMIGO1, whereas the AMIGO1 cRNA rescues expression of AMIGO1 but not of HMGB1. All groups were compared by one-way ANOVA followed by Fisher’s LSD post hoc test and Bonferroni correction; *, p < 0.001. The error bars indicate the S.E. values.

C and D, both Hmgb1 and AMIGO1 cRNA have a rescue effect on the development of catecholaminergic neural networks of telencephalon in the HMGB1 morphant. C shows statistics of larvae with positive TH1 staining in the 5-dpf larval telencephalon. Four independent injections were carried out, and 10 larvae from each group (50 larvae per injection group) were randomly selected for detection. >90% of the HMGB1 morphants have no TH1-positive cells in telencephalon. The rescue effects of Hmgb1 or AMIGO1 cRNA coinjection with HMGB1 MO1 are from >50% to even more than 70%, whereas the GFP cRNA does not display a significant rescue effect. The groups were analyzed by one-way ANOVA followed by Fisher’s LSD post hoc test and Bonferroni correction; *, p < 0.001. The error bars indicate the S.E. values. D shows a ventral forebrain view of 5-dpf larvae immunostained with anti-TH antibody, which was used as the basis of counting the larvae with positive anti-TH immunostaining in the telencephalon. The HMGB1 MO1 morphant has no catecholaminergic networks in telencephalon (arrow). Partial rescue in the development of the catecholaminergic pathways is seen in the Hmgb1 and AMIGO1 cRNA-injected groups compared with the uninjected or the 5mis MO-injected larvae. The stack size is about 100 μm throughout the brain thickness, and the step interval is 1 μm. Di, diencephalon; Po, preoptic region; Tel, telencephalon. Scale bar, 80 μm.
cell-to-cell signaling within the niches that regulate neural progenitors. It is currently generally accepted within the HMG field that HMGB1 can be actively secreted upon cell stimulation by cytokines/growth factors, and its secretion can be even induced by cell contact with extracellular matrix (for review, see Ref 2). Because the stem cells and progenitor cells reside within niches that are areas where many types of cytokines/growth factors and matrix factors regulate cells, one would expect HMGB1 to become secreted on such areas. In fact, in whole-mount immunostaining, HMGB1 can be detected in wild-type but not in knockdown zebrafish larvae at the plasma membrane level, resembling a matrix-type structure that surrounds the cells. We have previously shown that HMGB1 can regulate neural cells and other cell types in vitro as a cell matrix-associated molecule (for review, see Ref 2), which would be consistent with a role as a factor surrounding neural progenitors and regulating them through transmembrane signaling. However, one should keep in mind that HMGB1 may have important intracellular functions in neural progenitor regulation, a possibility that clearly warrants further studies. In the immune cell regulation, HMGB1 was recently shown to participate in both extracellular and intracellular mechanisms (39).

HMGB1 has several cell surface receptors, and further work is warranted to elucidate the relative importance of different membrane receptors of HMGB1 in neural progenitors. Regulation of many cell types by HMGB1 is generally suggested to depend, at least partially, on binding to RAGE. A search of the zebrafish genome data base does not identify any obvious RAGE homologue, although hypothetical protein structures displaying some similarity in the domain structure (for example, an Ig domain structure with ~30% similarity compared with the HMGB1 binding Ig domain of mammalian RAGE) can be found in the data base. Interestingly, Toll-like receptors that were initially identified as proteins guiding neuronal development in Drosophila have been recently implicated in neurogenesis in mouse brain (40). Toll-like receptors have been identified as HMGB1 receptors in the immune system in several studies (for review, see Ref 2), and their possible role as HMGB1 receptors in brain development needs to be elucidated.

A search of genes regulated by HMGB1 in embryonic neural cells using ordered differential display analysis was the basis of cloning of a novel adhesion protein designated as AMIGO1 (20). In these in vitro studies regulation of AMIGO1 expression was shown to depend on extracellular matrix-bound HMGB1 and transmembrane signaling in embryonic neural cells. The finding that HMGB1 regulates AMIGO1 expression in a similar manner in zebrafish larvae provides further evidence for a role of HMGB1 as an extracellular factor that would mediate cell-to-cell communication within the niches that are required for survival/proliferation of neural progenitors.

**HMGB1, Pax6, and Wnt Signaling**—Of the regional markers of brain development analyzed in the current study, Pax6 expression is of particular interest in the HMGB1 knockdown zebrafish. Lack of HMGB1 clearly results in reduction of Pax6 expression, and may be involved in the mechanism of Pax6 in forebrain development (33, 38). Our finding of enhanced Wnt signaling in the HMGB1 knockdown may, therefore, be due to down-regulation of Pax6 activity.

Wnt signaling has a complex role from very early to late stages of the nervous system development (for review, see Ref. 41). In particular, Wnt8 has been reported to play a key role in brain development (42, 43) and to affect development of cat-
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