Improved osteoblast adhesion and osseointegration on TiO$_2$ nanotubes surface with hydroxyapatite coating

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To improve initial osteoblast adhesion and subsequent osseointegration, TiO$_2$ nanotubes layer was constructed on the titanium (Ti) surface by anodic oxidation (AO), with an additional hydroxyapatite (HA) coating to form the AO/HA surface. Tests on in vitro cellular activity displayed that the AO surface, especially the AO/HA surface, promoted initial adhesion, proliferation and differentiation of osteoblast cells. The modified AO and AO/HA surfaces further presented an up-regulated gene expression of osteogenic and adhesion markers collagen type I (COL), osteopontin (OPN), osteocalcin (OCN) and vinculin. In addition, in vivo experiments with a rat model demonstrated that the AO surface, particularly the AO/HA surface, achieved earlier osseointegration and a superior bone bonding ability compared with Ti. Our study shed light on a synergistic role played by nanotopography and HA in promoting osteoblast adhesion, proliferation, differentiation and osseointegration, thus suggesting a promising method for better modifying the implant surface.

Keywords: TiO$_2$ nanotube, Hydroxyapatite coating, Osteoblast, Adhesion, Osseointegration

INTRODUCTION

Titanium (Ti) and its alloys are used extensively in dental and orthopedic clinical practice because of their excellent biocompatibility, corrosion resistance and mechanical properties. However, the bioinertness of Ti impairs its bioactivity and rapid binding to bone cells and tissue. Rapid osseointegration serve as a critical factor that determines the implant success$^{11}$. In addition, initial cell adhesion plays a crucial role in regulating the early response cascade towards the implant, including cell proliferation, differentiation and ultimate osseointegration$^{22}$.

To improve the initial osteoblast response and osseointegration of the implant materials, numerous methods have been used for Ti based implant surface modification$^{8}$. Among the various approaches, anodic oxidation (AO) has recently attracted much attention$^{3,5}$. AO is an electrochemical method easily implemented to produce ordered, uniform and controllable nanostructured surfaces on a variety of metals and alloys, which is an economical, simple, and versatile technique$^{6}$. It is also reported that the TiO$_2$ nanotube structure produced by anodization exhibits beneficial effects on osteoblast cell proliferation and differentiation$^{7,8}$. In order to achieve better osseointegration, hydroxyapatite (HA) has also been combined to the aforementioned TiO$_2$ nanotube structure. HA is a widely-accepted chemical component which can promote bone formation, due to its chemical similarity to the inorganic component of human natural bone, excellent osteoinduction and biocompatibility to the surrounding bone tissues$^{9}$. Among the various methods of HA coating, high temperature used in the coating process of plasma spraying or flame spraying method caused poor prognosis because of dissolution or peeling of the coating layer$^{10}$. In order to avoid the disadvantages produced by high temperature, we employed a biomimetic method to deposit a biologically active HA layer on the anodized surface by immersion of the substrate in simulated body fluid (SBF) under physiological conditions$^{11}$. Although several studies have reported the interaction between SBF and Ti surfaces$^{12,13}$, currently there is little information available about the biological effects of biomimetically deposited HA coating on anodized TiO$_2$ surface.

In this study, we investigate the effect and mechanism of osteoblast adhesion and osseointegration on the anodized TiO$_2$ surface with a biomimetically deposited HA coating. Scanning electron microscope (SEM), X-ray diffractometer (XRD), and contact angle test were utilized to characterize the physicochemical properties of these modified surfaces. More importantly, SEM observation, immunofluorescence staining of cytoskeleton actin and adherent cell number counting were carried out to evaluate the effect of cell adhesion on different surfaces. In addition, MTT assay and alkaline phosphatase (ALP) activity test were performed to compare the cell proliferation and differentiation between pure Ti and modified surfaces. Additionally, gene expressions of collagen type I (COL), osteopontin (OPN), osteocalcin (OCN) and vinculin were analyzed to further investigate the underlying molecular mechanisms. Furthermore, the influence of the modified AO and AO/HA surfaces on early osseointegration was evaluated through a rat model in vivo. The aim and
originality of this paper lies in evaluating the biological effects of the modified TiO\textsubscript{2} nanotubes surface with biomimetically deposited HA coating and elucidating the possible molecular mechanisms. We hypothesized that the nanotopography and HA exert synergistic effects on promoting initial osteoblast adhesion and osseointegration, with increased expressions of adhesion and osteogenic differentiation marker genes.

**MATERIALS AND METHODS**

**Specimen preparation and characterization**

Commercially available pure Ti plates in the dimension of 10×10×1 mm were polished with #1000 SiC abrasive paper, and then ultrasonically cleaned in acetone, ethanol and deionized water for 5 min, respectively. The AO process was carried out in 1 M NaF solution under a constant voltage of 10 V at room temperature for 1 h. A graphite electrode served as cathode. The anodized sample was denoted as AO surface. For comparison, the as-polished Ti plate (PT) was used as the control group.

The prepared AO plates were soaked in 30 mL of SBF at 37°C without stirring for 2 weeks to prepare biomimetic deposited HA coating on the surfaces. The ionic concentrations in SBF are nearly equal to those in human body blood plasma, according to Kokubo’s research work\textsuperscript{14}. Through biomimetic method, a HA coating was deposited on the AO surface to establish the AO/HA surface.

Surface morphology and crystal structure were observed using SEM (Hitachi S-4800, Tokyo, Japan) and XRD (Rigaku D/max 2500, Tokyo, Japan). The hydrophilicity was assessed from measuring the contact angle of deionized water at room temperature. The representative images of three independent experiments are shown. Data are expressed as mean±standard deviation (SD), n=3.

**Cell culture**

The mouse derived MC3T3-E1 osteoblast-like cells (CRL-2593, ATCC, Rockville, MD, USA) were cultured in α-MEM medium (Gibco BRL, Grand Island, NY, USA) with 10% fetal bovine serum (Hyclone, Logan, UT, USA) and 3% penicillin/streptomycin. Cultures were maintained at 37°C in an incubator with a fully humidified atmosphere of 5% CO\textsubscript{2}.

**Cell morphology, cytoskeleton actin staining and adherent cell counting**

Osteoblasts were seeded on substrates (PT, AO, AO/HA) at a density of 2×10\textsuperscript{4} cells/mL and allowed to attach on the surfaces for 4 h. To observe the cellular morphology on the sample surface, the cells adhered on the sample surface were fixed with 2.5% glutaraldehyde in phosphate buffered saline (PBS, pH=7.4) for 4 h, and then rinsed with PBS twice, followed by dehydration in a grade ethanol series and critical point drying. After gold sputtering, the cell morphologies were observed by SEM.

After incubating with different substrates for 4 h, the MC3T3-E1 cells were rinsed twice with PBS and fixed with 4% paraformaldehyde at room temperature for 10 min. The cells were permeabilized with 0.1% Triton X-100 for 3 min and washed twice with PBS. The samples were first stained with Rhodamine Phalloidin (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), and then counterstained with 4',6'-diamidino-2-phenylindole (DAPI) for a further 5 min in the dark. The stained actin cytoskeleton (red) and cell nuclei (blue) were examined using a confocal laser scanning microscopy (CLSM; TCS SP5, Leica, Wetzlar, Germany). The overall cellular area to nucleus area (C/N ratio) was measured using the Image-pro Plus J software (ver. 5.0, Media Cybernetics, Silver Spring, MD, USA). Five different fields of view were photographed at random for each sample, and three separate samples were measured for each group.

After incubating with different surfaces for 4 h, the non-adherent cells were removed by rinsing with PBS. Cells were fixed and stained with DAPI (Invitrogen, Thermo Fisher Scientific) for 5 min. Five fields of view were imaged randomly for each sample and three different samples were calculated in each group. The substrates were imaged via an inverted fluorescence microscope (Olympus IX71, Tokyo, Japan) and cell numbers counting were performed using the Image-pro Plus J software mentioned above.

**Cell proliferation and ALP activity**

The cells were seeded on the substrates at a density of 2×10\textsuperscript{4} cells/mL. Then cell proliferation ability was investigated using the MTT colorimetric assay (Sigma, St. Louis, MO, USA). After culturing for 1, 2 and 4 days, the samples were washed and then MTT (5 mg/mL) was added to each well with continuous culture for 4 h. After removal of the culture medium, dimethyl sulfoxide (DMSO; Sigma) was added to each well and vibrated for 10 min. Finally, the absorbance of DMSO solution from each well was measured at a wavelength of 490 nm via a spectrophotometer (Elx800, BioTek, Winooski, VT, USA). The values from three independent experiments were used for calculation.

The MC3T3-E1 cells were seeded on different surfaces at a density of 2×10\textsuperscript{4} cells/mL. After 1, 2 and 4 days of incubation, cells were washed with PBS and then lysed in Triton X-100 (0.1%) through standard freeze-thaw cycles. The ALP activities of the samples were evaluated using an ALP activity kit (Sigma-Aldrich, Saint Louis, MO, USA). The ALP activity was normalized to the total protein amount measured by BCA protein assay kit (Thermo Scientific, Rockford, IL, USA). The experiments were repeated three times.

**Osteogenic gene expression analysis by quantitative real-time PCR**

Cells with a concentration of 2×10\textsuperscript{4} cells/mL were cultured for 1, 2 and 4 days. Then, total RNA was isolated from cells using the Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). Highly purified gene specific primers for COL, OPN, OCN, vinculin and
Table 1  Primers for real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene bank ID</th>
<th>DNA primer</th>
<th>Sequence</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>NM_008084.3</td>
<td>Forward</td>
<td>5’-GGTGAAGGTCGGTGTGAACG-3’</td>
<td>233</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>5’-CTCGCTCCTGGAAGATGGTG-3’</td>
<td></td>
</tr>
<tr>
<td>COL</td>
<td>NM_007742.4</td>
<td>Forward</td>
<td>5’-TAAGGGTCCCCCAATGGTGAGA-3’</td>
<td>203</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>5’-GGGTTCCCCTCAGCTCTACAT-3’</td>
<td></td>
</tr>
<tr>
<td>OPN</td>
<td>NM_001204203.1</td>
<td>Forward</td>
<td>5’-CTCACATGAAGAGCGGTGAG-3’</td>
<td>174</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>5’-TCTCCTGGCTCTTCTTTGGA-3’</td>
<td></td>
</tr>
<tr>
<td>OCN</td>
<td>NM_007541.3</td>
<td>Forward</td>
<td>5’-GGACCATCTTTCTGCTCAGCTG-3’</td>
<td>131</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>5’-GTTCACTACCTTATTGCTCTCCTG-3’</td>
<td></td>
</tr>
<tr>
<td>vinculin</td>
<td>NM_009502.4</td>
<td>Forward</td>
<td>5’-GATGCTGGTGAACTCAATG-3’</td>
<td>171</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>5’-CGAATGATCTCGTAAATCTC-3’</td>
<td></td>
</tr>
</tbody>
</table>

the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were purchased from Sangon Biotech company (Shanghai, China). The primer sets along with the length of the resulting amplicons and the GeneBank accession number were shown in Table 1. Real-time PCR analysis was examined in three independent experiments. The quantitative real-time PCR analyses were performed as mentioned in a previous article. Amplification was conducted in triplicate using one cDNA sample for each interested gene. The most representative result of three separate experiments is shown.

Animal experiment
This study was carried out in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The ethical aspects of the animal experiment was approved by Animal Ethical and Welfare Committee (AEWC) of Tianjin Medical University (Approval No. TMUaMEC 2016006). Sixteen weeks old male Sprague Dawley rats (450±10 g) were randomized into PT, AO and AO/HA modified groups (n=5). The cylindrical implant rods (diameter of 2 mm, length of 2 mm) were separately implanted into the femora of rats and harvested 5 weeks after implantation.

Histological analysis and push-out test
The harvested rat femora were further fixed and dehydrated with grade ethanol series. Undecalcified tissue blocks were processed in ascending concentration of Technovit 7200 VLC (Heraeus Kulzer, Wehrheim, Germany) and embedded in a fresh solution of the same resin. After section preparation, hematoxylin and eosin (H&E) staining was performed and the sections were observed under microscope (Nikon Ni-E, Tokyo, Japan).

Then, the bonding strength at the bone-implant surface was assessed by biomechanical push-out test. Briefly, the fresh femora containing implants (n=5) were collected and the electromechanical testing machine (Instron 5544, Canton, MA, USA) was used for tests.

RESULTS
Surface topography and wettability
The SEM results showed that, the as-polished Ti surface displayed an even and smooth surface (Fig. 1a). After anodic oxidation, homogeneous and uniform array of nanotubes was formed. The inner diameter of the nanotubes was approximately 70 nm (Fig. 1b). Then, after biomimetic deposition, a new layer of substance grew on the TiO2 nanotubes, partly covered the anodized nanotube arrays (Fig. 1c).

The testing machine equipped with a pushing rod (0.8 mm diameter) and a 0.5-kN load cell was used to load the implant vertically downward with a displacement speed of 1 mm/min. The push-out value was determined by detecting the peak of a load-displacement curve.

Statistical analysis
The data in the present study were analyzed using SPSS 14.0 software (Armonk, NY, USA). A one-way ANOVA followed by the Student-Newman-Keuls post hoc test was used to evaluate the statistical significance of the difference between samples. Differences with p<0.05 were considered to be significant.

RESULTS
Cell adhesion
SEM results displayed that after 4-h incubation, the adherent cells are nearly spherically shaped on the PT surface (Figs. 2a, d); while on the AO surface, cells begin
to spread out, with several filopodia and lamellipodia extending into the nanotubular architecture (Figs. 2b, e). Remarkably, on the AO/HA sample, osteoblast cells exhibited a well-spread morphology, with many well-developed filopodia and lamellipodia of cell extensions protruding into the underlying nanotubular architecture (Figs. 2c, f).

The cytoskeletal images further revealed that after 4-h incubation, cells on the PT surface displayed less actin filament formation; while cells exhibited
more stretched actin filaments on the AO surface. It is noteworthy that cells on the AO/HA surface displayed many actin filaments spreading out with random orientation, indicating good cell communication (Fig. 3A). It suggested a better biocompatibility of the AO surface and AO/HA surface than the control. In addition, we further investigate osteoblast adhesion behavior quantitatively via image analysis. As shown in Fig. 3B, the total cellular area to nucleus area (C/N ratio) on the AO surface was 1.5-fold of that on the pristine Ti substrate ($p<0.05$). In particular, further increased C/N ratio was observed on the AO/HA surface, which was 1.8-fold of that on the pristine Ti surface ($p<0.05$). These results reveal that cytoskeleton organization was enhanced on the AO surface, especially on the AO/HA surface, compared to original pure Ti substrate.

Moreover, Fig. 3C showed that the adherent cell numbers per field of view on the AO and AO/HA surfaces are 1.7-fold and 2-fold of that on the PT surface, respectively ($p<0.05$). Furthermore, the number of cells on the AO/HA surface is 1.2-fold of that on the AO surface, with statistical significances ($p<0.05$). These results indicate that the AO surface promotes initial cell adhesion, while the AO/HA surface further enhances the osteoblast adhesion, probably due to the synergistic effects rendered by the nanotopography and HA coating.

**Cell proliferation, differentiation and quantitative real-time PCR**

Cell proliferation was evaluated by MTT assay and a progressive increase for all three surfaces was observed over the 4-day period. At each time point, increased cell proliferation is shown on the AO and AO/HA surfaces,
compared to the PT surface. In addition, the AO/HA surface was more favorable for cell proliferation than the AO surface (Fig. 4A, p<0.05).

As shown in Fig. 4B, both AO and AO/HA surfaces elicited a significant up-regulation of ALP activity over the flat Ti during the 4-day culture period. In addition, the AO/HA surface induced higher ALP production than that of the AO surface (p<0.05).

The gene expression levels of COL, OPN, OCN and vinculin are all upregulated on both AO and AO/HA surfaces than the PT surface, increase with the incubation time. In particular, the AO/HA surface showed significantly increased gene expression levels than the AO surface (Fig. 5, p<0.05). These results indicate that the AO surface, particularly the AO/HA surface promoted osteogenic proliferation, differentiation and osteogenesis-related gene expressions in the osteoblast cells.

Fig. 4 MTT assay (A) and ALP activity (B) representing the MC3T3-E1 cell proliferation and differentiation levels on the PT, AO and AO/HA surfaces. Data are expressed as mean±SD (n=3). * and # indicate statistical significance p<0.05 vs. PT and AO groups, respectively.

Fig. 5 Real-time PCR results representing the MC3T3-E1 cell gene expression levels on the PT, AO and AO/HA surfaces. Data are expressed as mean±SD (n=3). * and # indicate statistical significance p<0.05 vs. PT and AO groups, respectively.

Fig. 6 Representative H&E staining images at the bone-implant interfaces of PT (a), AO (b) and AO/HA (c) surfaces after 5-week implantation. CNT: connective tissue; B: bone; scale bar, 200 µm. (d) shows the push-out tests results. Data are expressed as mean±SD (n=5). * and # indicate statistical significance p<0.05 vs. PT and AO groups, respectively.
In vivo experiment
H&E staining reveals that a thick layer of connective tissues was found around the PT surface, with some osteoid loosely attached to the implant surface (Fig. 6a). While relative dense bone trabeculae were observed around the AO substrate, with only a thin layer of connective tissues remaining (Fig. 6b). In particular, well-formed mature bone tissue had grown energetically around the AO/HA surface, with only sparse connective tissue left (Fig. 6c). Furthermore, push-out test showed that the bonding strengths of AO and AO/HA surface were 59±7 N and 96±8 N, respectively, compared to that of polished Ti (24±9 N, p<0.05, Fig. 6d). These results demonstrated excellent osseointegration for AO and AO/HA groups.

DISCUSSION
Ti implant surface modification to enhance osseointegration represents a continued challenge in the implant design[16]. In the current study, a HA coating on anodized Ti surface was produced. SEM and XRD results verified the expected changes in surface topography and composition, indicating the successful preparation of the surface. Moreover, the contact angle test results suggest that the initial hydrophobic Ti surface was changed to a hydrophilic surface by AO and the HA coating addition. Moreover, the contact angle test results suggest that the initial hydrophobic Ti surface was changed to a hydrophilic surface by AO and the HA coating addition.

It is well-known that successful osseointegration relies on the stable initial adhesion of bone-forming cells (or osteoblasts) onto implant surfaces. Our results displayed that the anodized Ti surfaces, especially the AO/HA surface, are much more favorable than the as-polished surface to cell attachment and spreading, which agrees with previous finding[17,18]. It is well-established that increasing hydrophilicity improves bioactivity of biomaterials and so as to promote cell adhesion[19]. In our study, the improved surface wettability may explain the increased cell adhesion and cytoskeleton development observed on the AO surface and AO/HA surface, compared to the pristine Ti surface. The combination of nanostructure and HA may generate synergistic effects on improved initial cell adhesion.

In addition to cell adhesion, proliferation is also an important issue for discussion. In this article, MTT assay was used to investigate the cell proliferation ability. Our results revealed that the surface topography of nanotube significantly promoted osteoblasts proliferation, and the addition of HA to the nanostructured AO surface leads to further enhanced cell proliferation as reported previously[19,20]. Cell differentiations were evaluated by ALP activity assay. It is widely accepted that ALP is an early stage marker of osteoblast differentiation, and high levels of ALP accelerated cell differentiated into the osteoblasts and osteoblasts maturity[21]. The increased MTT value and ALP activity on the AO and AO/HA surfaces revealed the synergistic effects of nanostructure and HA in promoting cell proliferation and differentiation.

In addition, we also investigated the gene expression by real-time PCR analysis. The osteoblast cell adhesion stage, which usually happened within the first several days after cell seeding, plays an important role in regulating cell behaviors and subsequent bone formation[22,23]. Moreover, it has been reported that during the initial period of the differentiation process (days 1–4), osteoblasts become confluent and exit the cell cycle, with increased ALP expression[24]. Thus, in this article, we focused on the gene expressions of osteoblast cell adhesion and differentiation markers during the initial 4 days to explore the underlying molecular basis of mechanism. Further studies are needed to investigate the gene expressions of osteoblasts at later stages.

Clinical success of implants largely depends on early adhesion and osteogenesis of osteoblastic cells, which is regulated by related genes and protein[25,26]. Among them, vinculin serves as a membrane cytoskeletal protein which stabilizes focal adhesion (FA) and promotes integrin clustering and enlargement[27]. Previous studies revealed that nanotopography formed by AO induces higher cell adhesion in osteoblast cells[28]. Calcium and phosphate ions also induced a larger amount of vinculin in osteoblasts[29]. Similarly, our study revealed a sustained up-regulated gene expression of adhesion marker vinculin in the AO and AO/HA surfaces. Moreover, the AO/HA surface elicited a much higher vinculin gene expression than the anodized surface.

In addition, higher expression levels of genes related to cell differentiation may facilitate better osseointegration at the tissue-implant interface. Collagen (COL), a well-known early osteoblastic differentiation marker, is also the main component of the organic protein phase during early osteoid formation[30]. OPN serves as a middle-stage marker of osteogenic differentiation, and is associated with the onset of ECM mineralization at later time points[31]. OCN is a late-stage marker of osteoblast differentiation[32], and its production denotes the onset of mineralization[33]. In line with previous reports[31], our study revealed a continued up-regulation of three typical osteoblast differentiation marker genes (COL, OPN and OCN) on AO surface at different stages of osteogenesis. In addition, the genes were further upregulated on the AO/HA surface, validating the combined effect of nanotopography and HA coating at the genetic level.

To further explore the osteogenic potential of Ti, AO and AO/HA substrates in vivo, we inserted implant with different surfaces into rat femora to investigate the osseointegration properties. It has been reported that surface characteristics including physical topography and chemical components are identified as important factors affecting the subsequent osseointegration[34,35]. Implant surface with TiO2 nanotube arrays exhibited enhanced osseointegration and improvement of stability[36]. In particular, several previous studies have revealed higher bone-implant contact and gene expression levels of OCN, COL and ALP in the bone attached to 70-nm-diameter TiO2 nanotubes[37,38]. It is also reported that HA could potentially improve osseointegration[39,40], and Ca2+ and PO43- ions provided necessary substances for osteoblast maturity and
mineralization\textsuperscript{35}. Similar to previous reports, our results from \textit{in vivo} animal experiment revealed that both AO and AO/HA surface were much compatible with surrounding tissue than pure Ti surface. Especially, the AO/HA surface demonstrated superior bone formation ability. The advanced growth of new bone tissue and higher bonding strength between the AO/HA surface and surrounding bone tissue might be attributed to the combined effects of nanotopography and HA coating, which confirmed the \textit{in vitro} results.

CONCLUSION

In the present work, we fabricated TiO$_2$ nanotubes layer with biomimetically deposited HA coating. Our results revealed that during the initial stage of cell-tissue interaction, as early as the first 4 days after cell seeding, the newly formed hydrophilic AO/HO composite surface structure significantly enhanced initial cell adhesion, with improved attachment and spreading, which is due to the combined effects of nanotopography and HA coating. In addition, we discovered that the anodized Ti surfaces, especially the AO/HA surface could induce much higher osteoblast proliferation and differentiation \textit{in vitro}, with elevated gene expressions of adhesion (\textit{vinculin}) and differentiation (\textit{COL}, \textit{OPN} and \textit{OCN}) markers than the as-polished surface. Furthermore, \textit{in vivo} experiment also confirmed the superior osteogenic ability of the AO/HA modified surface at the interface of implant/bone than single AO surface or the original Ti substrate. Consequently, the results of present study proved our hypotheses that nanostructure and HA coating synergistically promote osteoblast adhesion, osseointegration and up-regulate gene expressions of adhesion and osteogenic differentiation markers. From the above observations, we propose that the combination of nanotube topography and HA deposition accelerated the initial cell adhesion and early osseointegration, which showed a very promising applicable future.

ACKNOWLEDGMENTS

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