**Quiescent Pluripotent Stem Cells Reside within Murine Peripheral Nerves that Can Be
Stimulated to Proliferate by rhBMP-2 or by Nerve Trauma**

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ABSTRACT

Background The clinical use of recombinant human bone morphogenic protein 2 (rhBMP-2, Infuse®) has been associated with nerve related complications including new-onset sciatica, and retrograde ejaculation

Purpose To better understand the interaction of rhBMP-2 and peripheral nerves with the intent of making procedures safer

Study Design/Setting Using a mouse model to examine the direct effect of diluted rhBMP2 (Infuse®) on murine sciatic nerves

Methods Animal studies were approved by the institutional IACUC. Balb/c mouse sciatic nerves were surgically exposed and 60ng (in 10µl) of rhBMP-2 was applied to the nerve. In

1 separate experiments, the sciatic nerves were subjected to mechanical compression using
2 forceps (and not exposed to rhBMP2). The third group of mice received direct injection of the
3 same amount rhBMP-2, or sterile saline as a control, into the hamstring area of the posterior
4 thigh without surgery. Mouse limbs with intact sciatic nerve were collected at 24, 48, or 72
5 hours after treatment for histology processing. A separate set of identically treated sciatic
6 nerves were retrieved from mice at the same time points and cells were isolated by collagenase
7 and trypsin digestion. The isolated cells were cultured in a stem cell medium containing 20%
8 knockout serum and human leukemia inhibitory factor. Immunohistochemical or immuno-
9 fluorescent cell stains against KLF4, Sox2, c-Myc, and Oct4 were performed on the mouse tissue
10 sections and cell culture slides. In addition, real-time PCR was performed to quantify the mRNA
11 expression profiles of the stem cell marker genes in cultured cells.

12 Results Profound morphological changes of the mouse sciatic nerves were noted after
13 exposure to rhBMP-2, with a rapid and robust cell proliferation within the nerves followed by
14 migration of these cells into surrounding tissue. Immunohistochemical stain revealed strong
15 nuclear stains of KLF4, Sox2, Oct4, and c-Myc on the overwhelming majority of these
16 proliferating cells in the nerve. Intramuscular injections of rhBMP-2 or willful physical
17 compression of the nerves showed similar cell proliferation effects as the direct application of
18 Infuse® to the sciatic nerve. The cells in stem cell culture medium grew steadily without feeder
19 cells and appeared fairly uniform. They were adherent to substrate and were motile. Double
20 fluorescent staining on the cells indicated co-localization of all pairs of the 4 stem cell markers
21 in the cell nuclei. Real-time PCR confirmed the strong mRNA expressions of KLF4, Sox2, Oct4,
22 and c-Myc in these isolated cells.

23 Conclusion Exposure to BMP-2 causes a marked proliferation of previously quiescent cells
24 within peripheral nerves. These cells simultaneously express KLF4, Sox2, Oct4, and c-Myc, the
25 transcription factors that confer embryonic pluripotency. Work described in the companion
26 paper reveals some of the differentiation capacity of the cells and their likely clinical
27 significance. In addition, the effects of direct exposure of nerves to rhBMP-2 as described here
28 should clearly illuminate the mechanism of BMP2 related nerve complications. We would
29 suggest that the use of this agent in proximity to known neural structures should only be done
30 with extreme caution.

31
32 **Keywords:** rhBMP-2, pluripotent stem cells, peripheral nerve, mouse, stem cell markers
33

1 INTRODUCTION

2 Bone morphogenetic proteins (BMPs), first discovered by Marshall Urist [1-3] are a cluster of
3 powerful cytokines with an ancient lineage. Recombinant human BMP-2 (hrBMP-2) has been
4 successfully manufactured and marketed (Infuse®) for the induction of bone formation. It has
5 been widely and successfully used to promote bone formation in human spine [4-7], and more
6 recently in long bones [8-10]. There is abundant literature on the subject of the molecular
7 mechanism of the bone forming process with several published reports from Boden and co-
8 workers [11-13], suggesting that the molecule induces a process of membranous bone
9 formation, which generates bone without creation of cartilage intermediates. However, the
10 details of this bone formation process have remained unclear.

11 With increased popularity and widespread clinical use of BMP-2 (Infuse®), some undescribed
12 complications from the use of Infuse® were gradually recognized during clinical practice,
13 including such problems as anterior pharyngeal swelling, new onset sciatica, and retrograde
14 ejaculation [14-17]. Animal studies reported by Dmitriev and Lehman also suggested that when
15 used for spine fusion, BMP-2 may trigger some morphologic changes within the spinal cord and
16 nerve roots that would negatively impact neurologic recovery [14]. It is generally accepted now
17 that the clinical use of BMP should be done with these complications much in mind. Most
18 surgeons now take great care to avoid direct exposure of dura, nerve roots or the sympathetic
19 chain to the BMP-2 solution during its surgical use. This report describes a likely mechanism for
20 some of these complications, and further describes the discovery of a new population of
21 previously undescribed stem cells.

22 To better understand the molecular and cellular mechanisms of the BMP-2 related clinical
23 complications, the initial objective of this study was to reveal the biological changes when a
24 peripheral nerve is directly exposed to purified rhBMP-2. Using the commercially available
25 Infuse® product, we exposed murine peripheral nerves to small quantities of dilute hrBMP-2
26 (Infuse), and observed the effects on the nerves, which included the rapid proliferation of cells
27 within the exposed nerves. Further, the proliferating cells within the nerves after Infuse®
28 application were isolated for culture and characterized using immunohistochemical stains and
29 real-time PCR techniques.

30 As we will show, these isolated cells unexpectedly express the embryonic stem cell markers
31 KLF4, Sox2, c-Myc, and Oct4. Based on the Yamanaka Nobel Prize winning studies [18, 19], adult
32 cells induced in culture to express these 4 genes possess the wide differentiation potential of
33 embryonic stem cells. Such cells are often referred to as induced pluripotent stem cells or iPCs
34 [19] and may be created from adult differentiated cells by using retroviral vectors to induce
35 expression of these 4 genes. Identifying cells expressing these 4 genes in the present report is
36 a remarkable finding; as such cells are not thought to exist in adult mammals. The data

suggests that this is a previously unknown and un-described variety of pluripotent stem cells that can be obtained from adult animals without the use of retroviruses or any direct manipulation of genes. This current report characterizes these cells, and suggests that they may have uses in regenerative medicine. We suggest that they be referred to as Nerve Derived Adult Pluripotent Stem cells or NEDAPS cells.

MATERIALS AND METHODS

Exposure of murine sciatic nerves to rhBMP

All animal procedures were approved by the Institutional Animal Care and Use Committee. Total of 150 female BALB/c mice at 8-week of age were purchased from The Jackson Laboratory (Bar Harbor, ME) and acclimated to the facility for at least one week prior to commencement of the study. After anesthetized with intraperitoneal injection of 90 mg/kg ketamine, 8 mg/kg Xylazine, and 1-2% Isoflurane (nose cone), the right leg of each animal was shaved, disinfected with Povidone-Iodine and ethanol, and the sciatic nerve was surgically exposed. 60 ng of BMP-2 in 10 µl (INFUSE® Bone Graft, Medtronic Spinal and Biologics, Memphis, TN) was directly applied onto the exposed nerve. The BMP-2 dosage used in the experiments was determined by the results of preliminary experiments. The incision was sutured closed and the animals survived for 24, 48, or 72 hours until sacrifice for harvest of the nerve, by re-exposing it and excising it for histologic analysis or cell culture. Control mice underwent sham surgery without BMP2 exposure or physical sciatic nerve disturbance were used as controls.

A second group of mice were prepared, anaesthetized and the sciatic nerve exposed as above. Instead of applying BMP-2 to the sciatic nerve, about 10 mm segment of exposed nerve was manually compressed using a surgical forceps at 2-3 mm intervals along its length. These nerves were similarly harvested for the recovery of cells as described above.

Another group of mice were prepared and anaesthetized in an identical manner, but percutaneously injected into the hamstring area of the posterior thigh with an identical concentration and volume of Infuse®. A 25 gauge needle was advanced from a posterior entry to reach the midpoint of the femur, then retracted 3 mm to deliver the injection. The animals were sacrificed as described above and the entire lower limb was immersed in formalin for 48 hours, after which the hamstring muscle mass (containing the sciatic nerve) was excised and prepared for histological sectioning and staining. Untreated control nerves were also studied.

Nerve derived embryonic like (NEDAPS) stem cell isolation and culture

As will be described in detail below, the nerves that had been exposed to the rhBMP-2 were remarkable for a proliferation of cells within the nerve. We have termed these cells NEDAPS cells. Proliferating NEDAPS cells were isolated from surgically harvested mouse sciatic nerves according to a published protocols with modifications [20]. Briefly, sciatic nerve segments were minced to 1mm pieces in Phosphate Buffered Saline (PBS) and pelleted by centrifugation at 600xg for 5 minutes. The nerve tissue was then incubated at 37°C in 0.5ml of 0.2% (0.27 U/ml) collagenase (Worthington Biochemical Corp) in sterile Dulbecco's Modified Eagle Medium (DMEM) for 90 minutes, followed by addition of an equal volume of 0.05% trypsin-EDTA solution for 5 minutes with agitation. 300µl of heat-deactivated fetal bovine serum (FBS) was added to the mixture to stop the enzyme digestion. After filtration through a 100µm-sized mesh, the isolated cells were centrifuged down at 600xg for 10 min. The cell pellets were resuspended and distributed to 6-well culture dishes, or 4-well chamber-slide in DMEM (Gibco, Life Technologies), supplemented with 20% Knockout serum replacement (KSR, Gibco), 100µM MEM non-essential amino-acid solution (Gibco), 1x GlutaMAX™-I (Cat. no. 35050-079, Gibco); 55µM β-mercaptoethanol (Gibco), 20 ng/ml human leukemia inhibitory factor (LIF, Gibco), 100 U/ml penicillin (Invitrogen, Grand Island, NY), and 100 µg/ml streptomycin (Invitrogen). The cells were cultured at 37°C, 5%CO₂ atmosphere.

Immunofluorescence Staining and image acquisition

To characterize the NEDAPS cells, double staining against pairs of embryonic stem cell markers (KLF4, Sox2, Oct4, and c-Myc) were performed. Cells grown in the chamber slides were fixed with 4% paraformaldehyde for 30 minutes at room temperature, washed 3x with PBS, and blocked with 3% normal donkey serum and 0.1% Triton X-100. Primary antibodies include goat anti-KLF4 (R&D), goat anti-Sox2 (Santa Cruz), goat and rabbit anti-Oct4 (Abcom), and rabbit anti-cMyc (Santa Cruz). Secondary antibodies used were donkey anti-goat IgG conjugated with Alexa 488, and donkey anti-rabbit IgG with Alexa Fluor® 594 (Life Technologies). Cells were double stained with primary goat- and rabbit- antibody pairs (KLF-4 + c-Myc, Sox2 + c-Myc, KLF-4 + Oct4, and Sox2 + Oct4) for 60 minutes at 37°C. After PBS rinse, the cells were incubated in 1:200 secondary antibodies with distinct fluorescent wavelength for 30 minutes at 37°C, and coverslips were mounted onto the sections with DAPI Fluoromount G (Southern Biotech) which counterstains cell nuclei. Stained cells were viewed under a TCS SP5 II confocal laser scanning microscope (Leica Microsystems) and images acquired with the LAS Image Analysis software. Optical single sections were acquired with a scanning mode format of 1024x1024 pixels, with a pixel size of 0.21 µm. Acquisition of automated-sequential collection of multi-channel images was performed in order to reduce spectral crosstalk between channels, and individual images of double staining signals were overlaid to generate co-localized images.

Real-time Polymerase Chain Reaction (RT-PCR) Test

KLF-4, c-Myc, Sox2, and Oct4 gene expression profiles of the cells extracted from the sciatic nerves were determined using RT-PCR techniques as detailed previously [21]. Briefly, cells were homogenized in a 5-ml Dounce homogenizer in STAT-60™ (Tel-Test, Friendswood, TX) solution and the total RNA was isolated by chloroform separation and isopropanol precipitation. Complementary DNA (cDNA) was reverse transcribed from 0.5 µg of total RNA in 40 µL PCR buffer containing 5.5 mM MgCl₂, 500 µM each of deoxynucleotide triphosphates, 0.5 U/µL RNase inhibitor, 2.5 µM random hexamers, and 1.25 U/µL reverse transcriptase (Perkin-Elmer Cetus, Norwalk, CT) on a Veriti 96-well Thermal Cycler (Applied Biosystems, Foster City, CA) at 25°C for 10 minutes, 48°C for 25 minutes, and 95°C for 5 minutes. RT-PCR reaction mixtures contained SYBR Green PCR Master Mix (Applied Biosystems), 2 µL cDNA, and 400 nM tested gene primer pairs were run in a StepOnePlus® Real-Time PCR System (Applied Biosystems) for 40 cycles. The fluorescent signals were dynamically recorded. The primer pairs for each target gene were designed using Primer3 program (<http://bioinfo.ut.ee/primer3/>) [22, 23] and constructed by Sigma-Genosys (Woodlands, Texas), while the sequences are detailed in Table I.

Statistical Analysis

At least of 10 mice per treatment group was examined for this study. Immunohistochemical or immune-fluorescent positive cells as well as comparative gene expression of the 4 transcription factors among groups were respectively compared by independent t-tests (IBM SPSS ver. 23.0). P < 0.05 was defined as statistical significant difference.

RESULTS

Figure 1a illustrates the sciatic nerve exposed during surgery. All mice sustained well the surgical procedure of exposing sciatic nerve and Infuse® application, and recovered from anesthesia within 2 hours. At sacrifice, there was no sign of wound infection.

Histological assessment suggested that the rhBMP-2 exposed sciatic nerves displayed evolving loss of continuity of the nerve sheath, and a proliferation of small, rapidly dividing cells at 24 and 48 hours after exposure (Figure 1 c - d), in comparison to the undisturbed controls (Figure 1b). Similarly, intramuscular injection of BMP-2 also resulted in dramatic morphological changes of the sciatic nerves. Profound changes in the integrity of the nerve, as well as the robust cellular proliferation, were readily observed similar the isolated nerves displayed in Figure 1. The tissue sections also reveal that the cells are seen to originate within the nerve, and swarm into the adjacent tissue where they are seen to migrate along the local tissue planes (Figure 2). More dramatically, immunohistochemical staining of the cells in culture confirmed ubiquitous existence of the cells expressing c-Myc (Figure 2A), Oct4 (Figure 2B, 2E), KLF4 (Figure 2C, 2F), Sox2 (Figure 2D), and Nanog (Figure 2G) after BMP-2 exposure. Notably, untreated nerves did not stain for these markers (Figure 2H, 2I, p<0.001). It was also obvious that the

involved nerves had acquired a very abnormal appearance after 24 hours: including nerve bundle edema, fiber rupture, and the proliferating cells that migrated out to surrounding tissues (Figure 2E, 2F, 2G, 72 hours).

Early results from some of the sham-operated control animals that did not have exposure to BMP2 also displayed a small level of cellular proliferation, and this was only seen in animals that had surgical exposures accompanied by more than normal bleeding, or inadvertent traction or compression of the nerve. Further investigation of willful compression of the nerves using blunt forceps, without BMP2 exposure, also resulted in a robust proliferation of NEDAPS cells. And over 90% of cells were KLF4, c-Myc, Oct4, and Sox2 positive, the same as the rhBMP2 treated nerves.

The cells isolated from the mouse sciatic nerves at 24 hours after BMP-2 exposures or mechanical insult as described above were found to attach to the culture plates within 2 days in the embryonic stem cell medium. The cells maintained the polygonal shape for multiple weeks and rarely formed small colonies. The growth and proliferation rate was relatively slow and its doubling time was about 72 hours. Notably, they did not require “feeder cells” to maintain their survival. Immunofluorescent double staining for various pairs of the 4 stem cell markers in the cell nuclei indicated that over 90% of the cells simultaneously expressed KLF4, c-Myc, Oct4, and Sox2 (Figure 3). The staining was universally seen in the cell nucleus, as would be expected for these transcription factors.

Real-time PCR results complemented the nuclear staining findings that significant mRNA over-expressions of KLF4, Sox2, c-Myc, and Oct4 were exhibited in RNAs from the NEDAPS cells obtained from both rhBMP2 exposure or mechanically compressed sciatic nerves. There were no statistical differences between the BMP-2 treated and physical compressed groups. Agarose gel electrophoresis of the PCR products visualized/confirmed the clean single band of each target gene (Figure 4).

DISCUSSION

BMP-2 has been widely used in spine fusion and other clinical conditions for bone growth. However, observations from our group and others have suggested that some clinical complications with the use of BMP-2 might be a result of its interaction with nerves. Indeed we do note that the nerves exposed to BMP2 under our experimental conditions often appear grossly abnormal within 24 to 48 hours including nerve bundle edema, fiber ruptures, and cell proliferation. We were interested to note that, the cellular proliferation that we have observed after either direct application of the rhBMP-2 solution to a surgically exposed sciatic nerve, or to intramuscular nerves after intramuscular injections of dilute rhBMP-2 do not result in bone

1 formation. Investigators in this field believe that either a sustained local release of BMP-2 or an
2 extremely high local dose is necessary for induction of bone formation after an intramuscular
3 injection in most cases (unpublished: S. Miller & P. Wooley, personal communication).

4 It seems that there is little doubt that the observed histologic changes in the current study are
5 related to the widely observed nerve related complications reported in patients treated with
6 rhBMP-2. We believe that we have likely illuminated critical features of the mechanism of how
7 the BMP-2 molecule stimulates bone formation, specifically, by inducing stem cell proliferation.
8 Importantly, identification of this new population of quiescent, apparently pluripotent stem
9 cells residing within peripheral nerves may have a much broader application to regenerative
10 therapies of many kinds.

11 Previous studies by others have indicated that BMP-2 plays a critical role in regulating the
12 proliferation and differentiation of mesenchymal stem cells such as bone marrow stromal cells
13 [24-27]. However, the cells identified and isolated from the sciatic nerves appear to have
14 differentiation potential beyond the mesenchymal lineages. A confocal fluorescence
15 microscope was used to co-localize the transcription factors in the same cell nuclei. Antibodies
16 against KLF4, Sox2, c-Myc, and Oct4 were paired in 6 combinations to ensure co-localization of
17 all 4 nuclear proteins in the cells. Figure 3 clearly shows the cells expressing all 4 of these
18 transcription factors (KLF4, Sox2, cMyc, and Oct4) associated with embryonic or pluripotent
19 stem cells [19]. Notably, the immunofluorescence was limited to the cell nucleus, as would be
20 expected for these transcription factors. To complement the immunofluorescent co-localization
21 of the stem cell markers at protein level, real-time PCR was performed to examine the mRNA
22 expression levels with specific target gene primers on the samples with or without mechanical
23 challenge or BMP-2 exposure. Significantly high expressions of KLF4, Sox2, cMyc, and Oct4 gene
24 copies and clean PCR product bands were obtained.

25 Early results from some of the sham-operated control animals that did not have exposure to
26 BMP2 displayed a small level of cellular proliferation, and this was only seen in animals that had
27 surgical exposures accompanied by more than normal bleeding, or inadvertent traction or
28 compression of the nerve. This finding encouraged the further study to examine the physical
29 compression of the surgically exposed sciatic nerves. Interestingly, these damaged nerves were
30 found to exhibit robust proliferation of NEDAPS cells with nearly identical numbers of cells as
31 the rhBMP2 treated nerves. The data suggested that either BMP-2 interaction or physical
32 challenge to the peripheral nerves may trigger the proliferation of the resting NEDAPS cells.

33 NEDAPS are also distinct from the stem cells produced by the manipulation of nuclear genes,
34 cells that are referred to as induced pluripotent stem cells or iPCs [18]. Although the cells that
35 we have identified and propagated fulfill the presently accepted gene expression based
36 definition of an embryonic stem cell (ie: expression of the 4 transcription factors), they have a

1 remarkably distinct appearance and behavior from previously described embryonic stem cells.
2 This would seem to be a new and very distinct class of stem cells. These findings stimulated us
3 to imagine that this remarkable process probably transcends the process of osseous healing.
4 We began to look at this as perhaps a very important mechanism by which the body can
5 generate stem cells for repair of tissue of a variety of types, when nerves in the area were
6 appropriately stimulated or, importantly, injured.

7 The discovery and characterization of human embryonic stem cells has opened the door to
8 many possibilities for human medical application [28, 29]. Such cells can make blastocyst like
9 structures, and have been induced to form microscopic “mini organs” in some cases. They are
10 not at this point in time particularly close to wide human application. Among the drawbacks is
11 the potential for these cells to form benign but very problematic tumors (Teratomas) [28].
12 Another problem is that these cells are not easily generated to be “individual specific”, and thus
13 have potential for immunologic rejection [30]. However, it has not escaped our attention that
14 what is very different about this newly discovered population of NEDAPS cells, is that they
15 appear to have a full differentiation potential, but do not have the obvious drawbacks of true
16 embryonic stem cells, and should not present any risk of teratoma formation, nor of immune
17 rejection if used for “autograft” treatments for the patient in which they arose. In the future, a
18 biopsy procedure on an expendable superficial nerve, followed by ex-vivo culture, may lead to
19 individual self-to-self stem cell treatments.

20 In summary, we hereby propose that there is a reservoir of quiescent stem cells (NEDAPS cells)
21 that reside normally within most if not all peripheral nerves. They may have many functions,
22 but this early work has suggested that they respond to signals which indicate local trauma. In
23 response to BMP-2 (as in signaling proximity to a fracture) the cells will proliferate and
24 differentiate into cells for bone repair. We further hypothesize that other signals will stimulate
25 differentiation into other tissue types. The presence of the “universal markers of pluripotency”
26 suggests that these NEDAPS cells will be capable of a wide variety of differentiation targets,
27 perhaps without vexing problems such as tumor formation or immune rejection. Further
28 studies are currently being carried out to characterize the differentiation potential of these
29 NEDAPS cells.

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FIGURE LEGENDS

Figure 1 (A) a macroscopic image of the mouse sciatic nerve exposed during surgery and ready for Infuse® application; (B) H&E stained section of on-disturbed mouse sciatic nerve; (C) 24 hours after rhBMP-2 exposure; and (C) 72 hours after rhBMP-2 exposure. (Magnification power of B – C: 40x)

Figure 2 Immunohistochemical staining on mouse sciatic nerve following rhBMP-2 challenge for 24 and 72 hours: specimens and stained nuclear proteins are labeled on each representative image. (2H) and (2I) were sciatic nerves without rhBMP-2 exposure as controls. (100x)

Figure 3 Immunofluorescent confocal microscope images revealing the double staining of the embryonic stem cell markers on the NEDAPS cells isolated from the BMP-2 challenged mouse peripheral nerves. Each row exhibits one set of the cells with co-localizations of two transcription factors with different wavelengths. DAPI was used to for nuclear staining. (640x)

Figure 4 Agarose gel images showing the real-time PCR products of NEDAPS cells. (M) 100bp DNA ladder; Lanes 1 -2 are cells isolated from BMP-2 exposed mouse sciatic nerves, and lanes 3 – 4 are cells from physical compressed nerves. Panel (A) = amplification products of Oct4; (B) = Sox2; (C) = c-Myc; and (D) = KLF4.