Semaphorin Profiling of Periodontal Fibroblasts and Osteoblasts

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ABSTRACT

Cells of the periodontal attachment (cementoblasts, osteoblasts, and periodontal ligament fibroblasts) are descended from a common progenitor (the cranial neural crest). During their differentiation into different cell types, these cells separate from one another to form a laminated structure. Semaphorins (and their neuropilins and plexin receptors) act as cell guidance molecules for other neural crest derivatives. It is predicted that the differential expression of these molecules will correlate with the ability of these cells to segregate. It is demonstrated that human pre-osteoblasts segregate from PDL and gingival fibroblasts in culture. In addition, these cells express different semaphorins and plexins. Semaphorins 3D and 7A were expressed preferentially in dermal fibroblasts, while semaphorin 6B was selectively expressed by pre-osteoblasts. Semaphorins 3B, 4C, 5B, and 6C and plexins B1 and C1 were expressed in reduced levels in pre-osteoblasts. Analysis of the data suggests that differential expression of semaphorins and plexins may be involved in regulating cell-sorting in the formation and regeneration of the periodontal attachment structure. Abbreviations: Periodontal Ligament (PDL), Reverse Transcriptase Polymerase Chain-reaction (RT-PCR).

KEY WORDS: neuropilin, plexin, osteogenesis, cementogenesis, differentiation.

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INTRODUCTION

The periodontal attachment of teeth is comprised of the periodontal ligament linking the radicular cementum to the alveolar bone of the tooth socket. This attachment structure is maintained by the presence of the adjacent mesenchymal gingival tissue. Thus, the structural determinants that maintain tooth position are composed of osteoblasts, cementoblasts, and periodontal ligament and gingival fibroblasts. Interestingly, all of these cells are derived from a common precursor, cranial neural crest cells of the first branchial arch (Le Douarin, 1999). Little is understood about the control of the differentiation of this embryonic cell population into its multiple adult phenotypes. Even less is understood about how these newly differentiated cells segregate and sort from one another to form the exquisitely stable and uniform laminated structure that is the periodontal attachment.

The regulation of neural crest cell migration is not fully understood (Bronner-Fraser, 1994, 2000); however, it is partly regulated by the expression of cell-cell and cell-extracellular matrix adhesion molecules (*e.g.*, CAMs, cadherins, and integrins). The regulation of these adhesive molecules involves localized cell-surface signals utilizing members of the ephrin and semaphorin families of molecules. Ephrin B1/Eph tyrosine kinase receptor interaction is involved with regulating the metameric patterning of neural crest cells in the thoracic region of chick embryos (Krull *et al.*, 1997). Semaphorin 3A/neuropilin receptor interactions are also involved in this process, as well as in the guidance of neural-crest-derived sensory axons (Eickholt *et al.*, 1999; Brown *et al.*, 2001; Feiner *et al.*, 2001).

Semaphorin (Collapsin) Structure and Function

Semaphorins are a family of cell-surface and secreted glycoproteins that influence axon guidance (Kolodkin *et al.*, 1993; Luo *et al.*, 1995). These molecules exist either as cell-surface transmembrane proteins, or as secreted proteins that associate with the cell surface. Many semaphorins appear to act as negative cues in axon guidance, and may be responsible for regulating the pathfinding of new growth cones, and possibly target selection (Kolodkin *et al.*, 1993; Luo *et al.*, 1995; Puschel *et al.*, 1995). Semaphorins have been associated with several functions, including the regulation of axon guidance, regulation of blood vessel patterning, and the regulation of leukocyte movement. Mice deficient in Sema3A expression display alterations in sensory neuron guidance, as well as skeletal and cardiac malformations (Behar *et al.*, 1996; Taniguchi *et al.*, 1997). In addition, Sema4D is involved in B-cell aggregation within the immune system (Hall *et al.*, 1996), and monocytes express Sema7A (Holmes *et al.*, 2002). Analysis of these data indicates that these molecules play a key role not only in proper neuronal guidance and target selection, but also in non-neural cell guidance and organogenesis.

Neuropilin and Plexin (Semaphorin Receptors)

Neuropilins and plexins act as cell-surface receptors for semaphorins. Neuropilin-1 has been identified as having significant binding affinity for Sema3A (He and Tessier-Lavigne, 1997; Kolodkin *et al.*, 1997; Nakamura

et al., 1998; Feiner *et al.*, 2001). A related protein, neuropilin-2, acts as a receptor for Sema3F (Chen *et al.*, 1997; Giger *et al.*, 1998). In addition, both neuropilin-1 and -2 were also demonstrated to bind other Class III semaphorins, Sema3C and Sema3E (Chen *et al.*, 1997, 1998). Similarly, hippocampal neurons expressing neuropulin-1 are sensitive to Sema3A, while others expressing neuropilin-2 are responsive to Sema3F (Chedotal *et al.*, 1998). Analysis of these data, taken together, indicates that neuropilins act as receptors for the Class III soluble/membrane-associated semaphorins.

Here we have chosen to investigate the role of semaphorins in regulating the formation of the laminated periodontal attachment apparatus. As a first step, we present evidence for the expression of most of the vertebrate semaphorins, neuropilins, and plexins in several adult periodontal mesenchymal cell types (osteoblasts, PDL, and gingival fibroblasts) as well as in dermal fibroblasts.

MATERIALS & METHODS

Tissue Culture

Periodontal and gingival fibroblasts were obtained from patients with healthy gingiva who underwent oral surgery at the Louisiana State University School of Dentistry for the purpose of removing impacted wisdom teeth. In all cases, tissues were obtained from subjects following informed consent as prescribed in an approved IRB protocol. PDL fibroblasts were obtained from the PDL remaining attached to extracted molars, while the gingival fibroblasts were obtained from loose gingival tissue that was free of epithelium and associated alveolar bone (Palaiologou *et al.*, 2001), and characterized in respect of transcripts (Lallier *et al.*, 2004). Cells were maintained in αMEM containing 10% fetal calf serum (FCS), 200 units/mL of penicillin, and 200 μg/mL streptomycin (GIBCO, Grand Island, NY, USA). PDL and gingival fibroblasts between the 5th and 12th passages were used. Four independent isolates of gingival and PDL

Table. Primers for Human Semaphorins, Neuropilins, and Plexins

Transcript	Forward Primer[a]	Reverse Primer	Length	Accession Number
Semaphorins				
β1 Integrin	CAAAGGAACAGCAGAGAAGC	GTGGAAAACACCAGCAGC	537	NM_002205
Sema3A[b]	ACAACATTCCTCAAAGCTCG	TCATTTCTTCCAGCAGACC	592	L26081
Sema3B	GGACCCAGGAAGGATAGAGG	AGAAGAEGGCATAGAGCAGC	515	U28369
Sema3C	GCAAAGATCCCACACACG	TTCCAGCAGAANCACATCC	623	AB000220
Sema3D	GCAAAGATACTGCATTCACTCG	ATTCTCCCATCATACTGCACC	532	XM094973
Sema3E	AAACCTGCCCATAAAAAACC	CACGTCCTTTCTGTACAAACC	613	NM012431
Sema3F	TGTGATGAAGCTACCAAAAAGG	GATGCCATAGATCAAATCCAGC	633	U52840
Sema4A	AACTTCCTGGGCAGTGAGC	TGAATTTCCCCTTAAAGACACG	469	AB029394
Sema4B	ACCCAGACACCCAAACAGC	AACAACGGAGGAAGGCAGG	590	XM044533
Sema4C	AATGCCAATGGTTACGTGC	ATAGCTCCAATGACTCACCAGG	575	NM017789
Sema4D	CAAAGATCGTCATCAACACG	TTCCCCCACTACAGAAAGG	764	U60800
Sema4F	CAGTACACATGACCCCATCC	CCACAGAGTGATGCCATCC	435	XM087234
Sema5A	TGTGATGAAGCTACCAAAAAGG	GATGCCATAGATCAAATCCAGC	633	XM004042
Sema5B	CCAACCATTTGCACTACAAGG	GAAAATCCAGAGCTACTGGAGC	713	XM032249
Sema6A	AACTTCATCAAGACGCACC	AACTTTCCCGAATCACTCC	745	AF279656
Sema6B	TAACCCCAGCGACATAAACG	TTAAACTCCATCGCAATCTCC	435	AF293363
Sema6C	CACCCAGACCAATAGCATCC	CCACAAAAAGCCTGTGACC	557	AB022434
Sema7A	TCATCAAAGCCACCATCG	AGCTCACATACAGCTTCCTCC	771	AF071542
Neuropilins				
NP-1	GCCTGACTCAAATCCTCC	ACACCATACCCAACATTCC	731	AF016050
NP-2	TCAACCCTCACTTTGAAATCG	TCCAGCCATTGTCATCACC	741	AF022859
NPESDN	CGTAATAACTTTTTGCCACC	GCTTCATTCCTTTCCACC	493	AF387547
Plexins				
Plexin-A1	CCTGAGAATGAGAATGCACC	ATCACGTTCACCCAGAAGC	734	XM051261
Plexin-B1	CTAATCGCACGCGAGATGC	CATCACACCAGAACCAGTCAGC	765	XM051491
Plexin-B3	ACCTCATGACGGAGATGACC	CAAGCGGCTATCATTCAGG	587	AF149019
Plexin-C1	AACCATTGCACTGCAAACC	GATTCCATCTTCAAGAATCACG	557	NM005761

[a] This Table lists the primers used for each of the transcripts to be examined by reverse transcriptase polymerase chain-reaction and identifies the predicted product length and NCBI Accession number for each sequence used.

[b] Sema = Semaphorin; NP = Neuropilin; NPESDN = Neuropilin-Endothelial and Smooth-muscle-cell-derived Neuropilin-like molecule.

fibroblasts were compared in this study.

Cell lines of human dermal fibroblasts (ATCC CRL-1502, CRL-1474, CRL-1489, and CRL-1497) and pre-osteoblasts (ATCC-CRL-11372) were obtained from the American Type Culture Collection. The dermal fibroblasts were isolated from the skin of both male and female neonates, children, adults, and embryos. The pre-osteoblasts are a line of fetal osteoblasts (obtained from a spontaneous miscarriage) that have been transfected with a temperature-sensitive expression vector (pUCCSVtsA58) and with the neomycin resistance expression vector pSV2-neo (Harris *et al.*, 1995). These cells were grown at 33°C to prevent their temperature-sensitive auto-differentiation. Pre-osteoblasts were maintained in DMEM/F12 (without phenol red) media containing 10% fetal calf serum (FCS), 200 units/mL of penicillin, and 200 μg/mL streptomycin (GIBCO, Grand Island, NY, USA). All of these cell types have been more fully characterized (Lallier *et al.*, 2004).

RNA Isolation and Reverse Transcriptase Polymerase Chain-reaction (RT-PCR)

RNA was extracted from gingival, dermal, and periodontal ligament fibroblasts with the use of guanidine thiocyanate, and gene expression was determined by RT-PCR (Palaiologou *et al.*, 2001). We chose the number of cycles by creating a standard curve using serial dilutions of DNA template known to include the genes in question, and determined those PCR conditions that reveal two-fold differences in gene expression over 3 orders of magnitude. From 25 to 35 cycles of PCR was determined to be within the linear range of detection for all of the genes examined. The primers for these studies were derived from the published DNA sequences for human semaphorins, neuropilins, and plexins (Table).

To determine the reliability of apparent differences in gene expression, we performed RT-PCR on 3 independent samples. The relative levels of transcript expression were determined by comparison of the PCR product intensities with a standard reference curve generated by PCR derived from serial dilutions of template. Relative expression is expressed as the ratio of the expression of a specific transcript by one cell type to that of another cell type. In this study, we determined that expression levels were significantly different if there was a five-fold or greater difference in expression.

Cell-sorting

Cell segregation was measured *in vitro* by means of Cell Tracker dyes (Molecular Probes, Eugene, OR, USA). Cells were grown to near-confluence, and labeled with 1 of 2 fluorescent dyes, by incubation in a PBS solution containing from 1 to 10 μM dye and 0.1% DMSO for 30 min at 37°C. The 2 dyes used were CellTracker Orange (5- and 6- [4-chloromethylbenzoylaminotetramenthyl-rhodamine] mixed isomers) and CellTracker Green CMFDA (5-chloromethylfluorescein diacetate). Cells were rinsed extensively with PBS, trypsinized to free them from their substrates, and triturated into a single cell suspension. Cells from different sources were labeled with different dyes and mixed at a ratio of 1:1. The mixtures of cells were then plated into multiple 60-mm tissue culture dishes and allowed to adhere for 1-2 hrs, before being fixed with 4% paraformaldehyde for 30 min, rinsed in PBS, and photographed under a Nikon Eclipse E600 fluorescence microscope (Tokyo, Japan). Matching dishes of cells were incubated for various times from 12-72 hrs before fixation and observation. For each experiment, 10 random, non-overlapping fields of view were photographed. The degree of cell segregation was determined based on the ratio of cell-cell contacts made by each cell within a field of view to each of its adjacent neighbors and was represented as the percentage of cells in contact with cells of similar type (similar color).

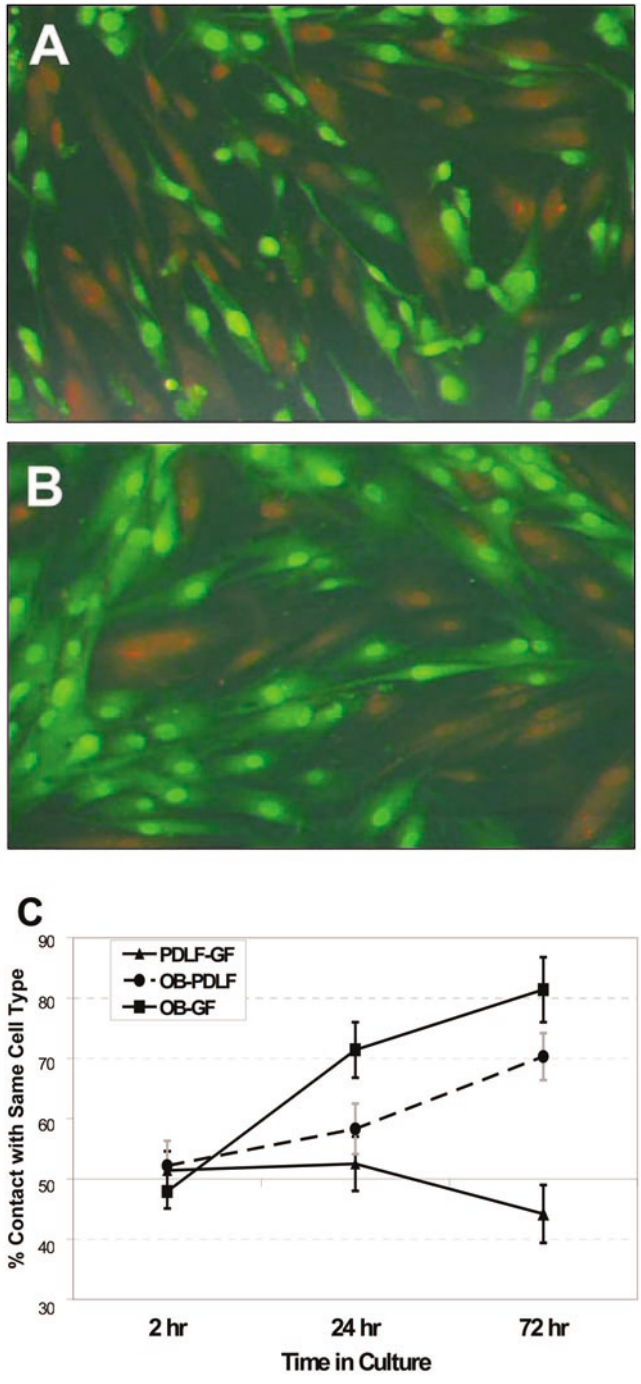
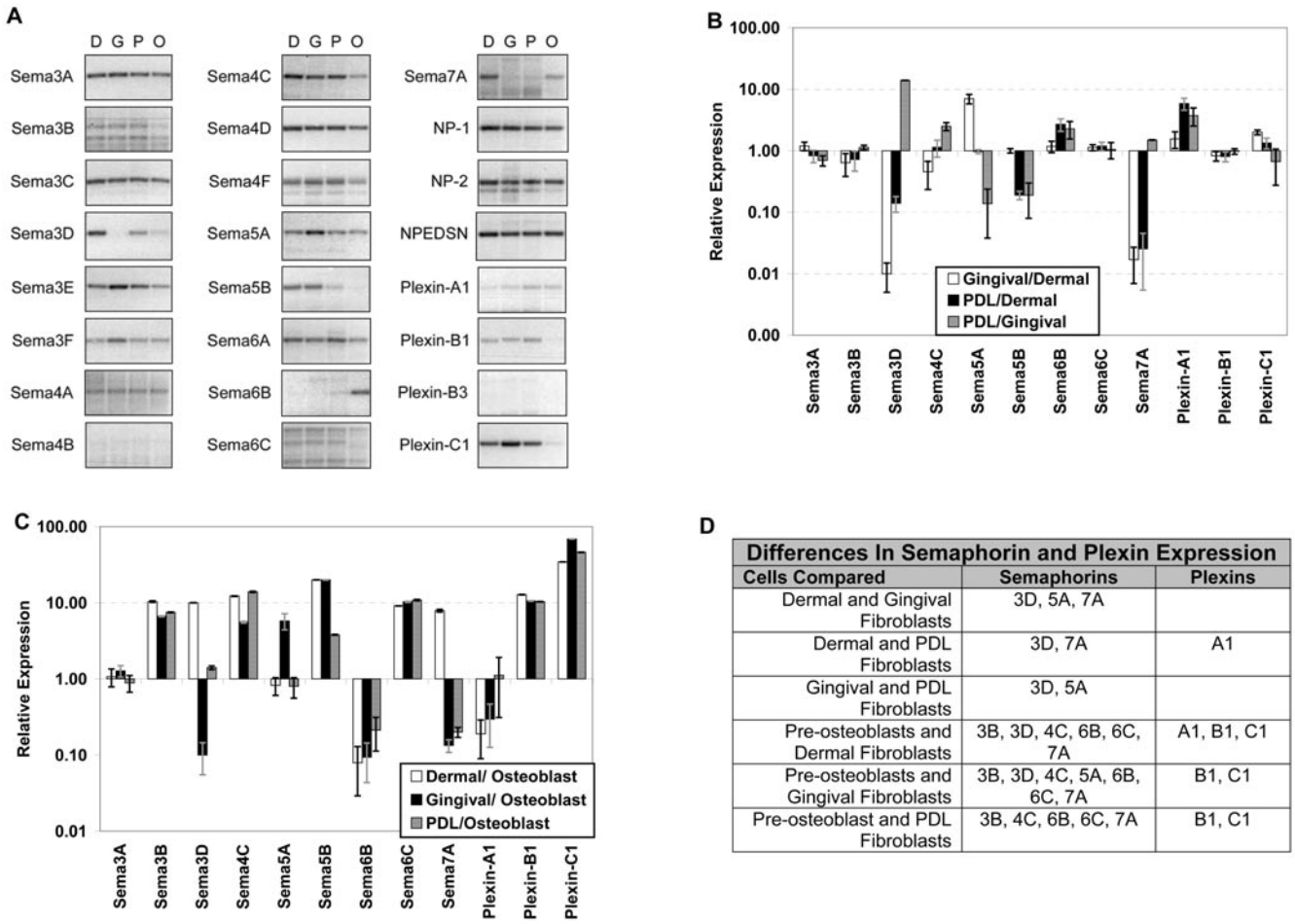


Figure 1. Periodontal cell segregation. Periodontal ligament (green) and gingival fibroblasts (red) were co-cultured and displayed little segregation after 24 hrs **(A).** In contrast, pre-osteoblasts (green) and gingival fibroblasts (red) displayed greater segregation when co-cultured for 24 hrs **(B)**. The percentages of cell contacts made by cells with either similar or dissimilar cell types (for periodontal ligament and gingival fibroblasts and pre-osteoblasts) were quantified and compared for cells co-cultured for 2, 24, or 72 hrs **(C).** Data points represent the mean and standard deviation of 3 independent experiments, each consisting of 10 non-overlapping microscopic fields containing at least 50 cells each.



D

Differences In Semaphorin and Plexin Expression		
Cells Compared	**Semaphorins**	**Plexins**
Dermal and Gingival Fibroblasts	3D, 5A, 7A	
Dermal and PDL Fibroblasts	3D, 7A	A1
Gingival and PDL Fibroblasts	3D, 5A	
Pre-osteoblasts and Dermal Fibroblasts	3B, 3D, 4C, 6B, 6C, 7A	A1, B1, C1
Pre-osteoblasts and Gingival Fibroblasts	3B, 3D, 4C, 5A, 6B, 6C, 7A	B1, C1
Pre-osteoblast and PDL Fibroblasts	3B, 4C, 6B, 6C, 7A	B1, C1

Figure 2. Expression of semaphorin, neuropilin, and plexin transcripts. **(A)** Reverse transcriptase polymerase chain-reaction (RT-PCR) analysis of transcripts expressed by gingival fibroblasts (G), dermal fibroblasts (D), periodontal ligament fibroblasts (P), and pre-osteoblasts (O). Relative RNA transcript levels for the semaphorins, neuropilins, and plexins were compared. The expression of the β1 subunit of integrin acts as a loading control, since it is expressed uniformly by all of the cells examined. The photo represents a typical result from 1 of 3 experiments. RT-PCR was performed for 25 cycles in this experiment. All RT-PCR reactions were repeated with 30 cycles for those transcripts that were initially detected poorly at 25 cycles. The PDL and gingival fibroblasts represented in panel (A) were isolated from the same individual. The expression of each transcript was compared between fibroblast populations **(B)** and fibroblast and pre-osteoblast populations **(C)**. Four independent isolates of dermal, PDL, and gingival fibroblasts were compared, and the comparison of expression was represented as the ratio of the mean transcript expression. Error bars represent the standard deviation. In general, a five-fold difference in expression was determined to be statistically significant by ANOVA analysis. **(D)** Those semaphorins and plexins with a greater-than-five-fold difference in expression are listed.

ELISA Assays

Semaphorin and plexin antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) were used for the detection of Sema3A (N-15), Plexin-A1 (S-16), and Olexin-C1 (N-17) protein expression. These antibodies were raised in rabbits and immunopurified to human peptides. Non-immune rabbit antiserum was used as a control, and antibody specificity was determined by peptide competition. Briefly, extracts of cell proteins (100 μg/mL in 50 mM PBS with 100 mM NaCl and 1 mM phenylmethylsulfonate) were plated into 48-well plates and allowed to adhere for 24 hrs at 4°C. Non-specific protein binding was inhibited with the use of 1 mg/mL BSA. A 10-μg/mL quantity of semaphorin- or plexin-specific antibody was incubated with each sample for 2 hrs at 4°C and probed with alkaline-phosphatase-conjugated secondary antibodies and visualized with thes use of 1 mg/mL p-nitrophenol phosphate (PNPP) in 0.1 M diethanolamine (pH 8.3) with 5 mM levamisole (incubated at 25°C for 30 min with gentle agitation). The enzymatic color reaction was stopped by the addition of 500 μL of 0.75 N NaOH, and the mixture was assayed for 405-nm absorbance in a microplate reader (FL600, BioTek, Winooski, VT, USA).

RESULTS

Sorting of Cells in Culture

Cells grown in culture displayed different potentials to segregate from one another (Fig. 1). PDL and gingival fibroblasts displayed little ability to separate in culture (Fig. 1A). In contrast, pre-osteoblasts displayed a greater tendency to separate from both PDL and gingival fibroblasts (Fig. 1B). This separation increased over time. In addition, gingival fibroblasts and pre-osteoblasts segregated more readily than did PDL fibroblasts and pre-osteoblasts (Fig. 1C). Thus, this *in vitro* model system for cell-sorting reflects the ability of pre-osteoblasts to segregate from adjacent mesenchymal cells *in vivo*.

Expression of Semaphorin, Neuropilin, and Plexin Transcripts by Fibroblasts and Pre-osteoblasts

The expression of semaphorin, neuropilin, and plexin transcripts was examined by semi-quantitative RT-PCR (Fig. 2A). Analysis of these data demonstrated that dermal, gingival, and PDL fibroblasts express many of these molecules in common (semaphorins 3A, 3B, 3C, 3D, 3E, 3F, 4A, 4B, 4C, 4D, 4E, 5B, 6B, and 6C; neuropilins 1, 2, and EDSN; and Plexin-B1 and Plexin-C1). These fibroblast populations varied from one another only in the expression of Sema3D, Sema5A, Sema7A, and Plexin-A1 (Fig. 2B). In contrast, pre-osteoblasts differed from all fibroblasts in their expression of Sema3B, Sema4C, Sema6B, Sema6C, Plexin-B1, and Plexin-C1 (Fig. 2C). In addition, pre-osteoblasts differ from at least one fibroblast population in Sema3D, Sema7A, and Plexin-A1. Sema3A and Plexin-A1 and Plexin-C1 protein expression mirrored their RNA expression (Fig. 3). In addition, intact PDL expressed the same semaphorin and plexin proteins as did PDL fibroblasts in culture, although at reduced levels. Therefore, pre-osteoblasts displayed greater differences in the expression of these cell-surface signaling molecules to fibroblasts than was seen between fibroblast populations. (Fig. 2D).

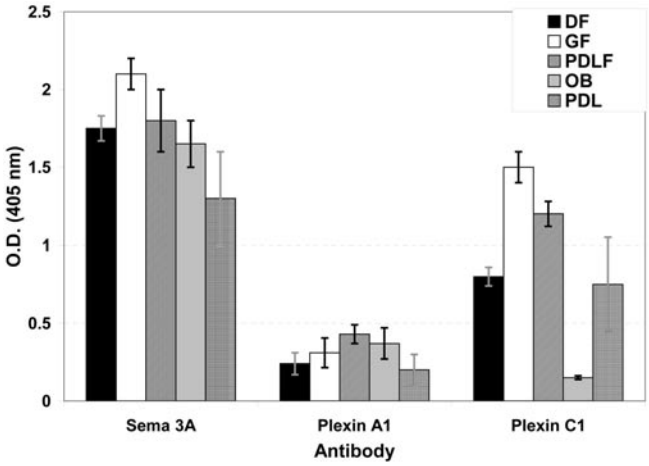


Figure 3. Semaphorin and plexin protein expression in periodontal cells. The expression of Semaphorin 3A, Plexin A1, and Plexin C1 in dermal fibroblasts (DF), gingival fibroblasts (GF), periodontal ligament fibroblasts (PDLF), pre-osteoblasts (OB), and intact periodontal ligament (PDL) was examined by ELISA. Each bar represents the mean and standard deviation for 4 samples each for 4 cell/tissue isolates (n = 16).

DISCUSSION

Semaphorins, along with their neuropilin and plexin receptors, are involved in the guidance of various cell populations, including neurons, blood vessels, and cells of the immune system. However, limited data exist on the expression of semaphorins, neuropilins, and plexins in the oral cavity. Sema3A, Sema3B, and Sema3C are expressed in dental epithelia and mesenchyme (Loes *et al.*, 2001). In addition, neural crest cells express neuropilin-1 (Eickholt *et al.*, 1999), and cardiac neural crest cells express Sema3C (Brown *et al.*, 2001; Feiner *et al.*, 2001). Our data correlate with all of these findings, with both neural-crest-derived fibroblast populations (gingival and PDL) expressing Sema3A, Sema3B, Sema3C, and neuropilins 1 and 2. Osteoblasts express Sema3A (Togari *et al.*, 2000) and neuropilins 1 and 2 (Harper *et al.*, 2001; Herzog *et al.*, 2001; Mayr-Wohlfart *et al.*, 2002). Analysis of our data confirms this expression pattern as well. In addition, our study demonstrates that these cells express a wide variety of semaphorins, neuropilins, and plexins (summarized in Fig. 2D). Thus, members of these three families of molecules are expressed in a highly complex pattern in oral mesenchymal tissues and represent potential regulators of tissue movements during periodontal development and regeneration.

Similarities between Gingival and PDL Fibroblasts

Analysis of our current data suggests a high degree of similarity between fibroblast populations. This is in stark contrast to previous data suggesting a greater degree of difference between these populations (Lallier *et al.*, 2004). In that study, PDL and gingival fibroblasts were more similar to each other than was either to dermal fibroblasts. In the current study, these same 3 populations varied only in the expression of 3 or 4 semaphorins and plexins (Fig. 2D). This may reflect an inherent difference between the transcripts being examined in each study. In the previous study, these fibroblast populations differed in their expression of transcripts involved with osteogenesis, indicating a potential difference in the developmental potential of these cell populations. In contrast, this study focused upon transcripts that may be involved in aiding cells in sorting themselves from their neighbors. Analysis of our *in vitro* cell-sorting data supports the finding that these cells do not segregate, and is supported by their similarity in semaphorin, neuropilin, and plexin expression. Analysis of these data, taken together, supports the supposition that these cells are functionally similar with regard to sorting, yet distinct in their osteogenic potential.

Similarities between Pre-osteoblast and Fibroblast Populations

While fibroblasts display great similarity to one another, these 3 cell populations display greater differences to pre-osteoblasts. Pre-osteoblasts express Sema6B to a greater degree than do all 3 fibroblast populations. In contrast, pre-osteoblasts also express Sema3B, Sema4C, Sema5B, Sema6C, Plexin-B1, and Plexin-C1 at significantly lower levels than do any of the 3 fibroblast populations. Pre-osteoblasts differed from dermal and gingival fibroblasts in their expression of 9 transcripts, and from PDL fibroblasts in the expression of 7 transcripts. This may reflect the ability of pre-osteoblasts to segregate from adjacent mesenchyme *in vivo*. This ability to segregate is mimicked by our *in vitro* assay system.

PDL fibroblasts display a greater ability to interact with pre-osteoblasts *in vivo*. This is reflected by the intermingling of collagen fibers (Sharpey's fibers) between ligaments and calcified tissue (bone or cementum). In our experiments, this is reflected by a smaller number of differentially expressed semaphorins and plexins between pre-osteoblasts and PDL fibroblasts (7) and gingival fibroblasts (9). This is mimicked in our *in vitro* assay system by the greater segregation seen between gingival fibroblasts and pre-osteoblasts. Thus, analysis of these data supports the potential role of semaphorin signaling in the segregation of pre-osteoblasts from the mesenchymal cell types of the developing and regenerative periodontal attachment apparatus. Further studies will be required to determine how these molecules function in this

process. In addition, the current study points out the extensive and complex pattern of expression of these families of molecules in a few distinct cell types. This could have broader implications, indicating a greater need to evaluate families of molecules in their entireties rather than focusing on the role of individual members of these families in isolation.

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