Calcineurin initiates smooth muscle differentiation in neural crest stem cells

Kris M. Mann, Jenna Lynn Ray, Edward S. Moon, Kristin M. Sass, and Mark R. Benson

Cardiovascular Research Center, University of Michigan, Ann Arbor, MI 48109

The process of vascular smooth muscle cell (vSMC) differentiation is critical to embryonic angiogenesis. However, despite its importance, the vSMC differentiation program remains largely undefined. Murine gene disruption studies have identified several gene products that are necessary for vSMC differentiation, but these methodologies cannot establish whether or not a factor is sufficient to initiate the differentiation program. A gain-of-function system consisting of normal vSMC progenitor cells would serve as a useful complement to whole animal loss-of-function studies. We use such a system here, namely freshly isolated rat neural crest stem cells (NCSCs), to show that activation of the calcineurin signaling pathway is sufficient to drive these cells toward a smooth muscle fate. In addition, we present data suggesting that transforming growth factor (TGF)-β1, which also causes NCSCs to differentiate into smooth muscle, activates calcineurin signaling in NCSCs, leading to a model in which activation of calcineurin signaling is the mechanism by which TGF-β1 causes SMC differentiation in these cells.

Introduction

Differentiation of vascular smooth muscle cells (vSMCs) is critical to arteriogenesis in the embryo. The first step in arteriogenesis is vasculogenesis, during which endothelial cells proliferate and form thin-walled tubules. Smooth muscle progenitor cells are then recruited to ensheath these endothelial tubules and are induced to differentiate into smooth muscle. The endothelial tubules are unstable and prone to regression until they are ensheathed by differentiated smooth muscle, so differentiation of smooth muscle is necessary for the development of stable, mature arteries (Carmeliet, 2000).

A comprehensive understanding of the mechanisms of smooth muscle differentiation has proven to be elusive, although a role in smooth muscle cell (SMC) differentiation has been proposed for several factors. For example, a variety of experimental results indicate that transforming growth factor (TGF)-β1 is involved in smooth muscle differentiation (Folkman and D'Amore, 1996; Perrella et al., 1998). Disruption of the endoglin gene, which encodes an endothelial cell surface protein that regulates TGF signaling, inhibits vascular smooth muscle differentiation (Li et al., 1999). Also, mice deficient for the activin-like kinase 1 TGF receptor also exhibit poor vSMC differentiation (Oh et al., 2000). In addition, diverse cell types differentiate into smooth muscle when treated with TGF-β1. These include bovine endothelial cells, the 10T1/2 line of murine embryonal fibroblasts, murine embryonic stem cells, and rat neural crest stem cells (NCSCs; Arciniegas et al., 1992; Shah et al., 1996; Drab et al., 1997; Hirschi et al., 1998). This finding strongly suggests that one of the normal, physiological effects of TGF-β1 is to induce SMC differentiation.

Several factors other than TGF-β1 are also thought to regulate smooth muscle differentiation. These factors include GATA-6 (Morrisey et al., 1996; Mano et al., 1999), CRP1 and CRP2 (Chang et al., 2003), dHAND (Yamagishi et al., 2000), and myocardin, which seems to act in concert with serum response factor (Wang et al., 2001; Du et al., 2003). However, the interplay between these factors in the differentiation process, and their relative importance to differentiation, is poorly understood. Most of the evidence that these factors are important is derived from loss-of-function analyses, chiefly murine gene disruption studies. These experiments provide valuable information but cannot answer certain important questions, like whether a given gene acts in a cell-autonomous manner or whether it acts proximally enough in the differentiation program to be sufficient to initiate smooth muscle differentiation in progenitor cells.

Address correspondence to M.R. Benson, 7301E MSRB III, 1150 W. Medical Center Dr., Ann Arbor, MI 48109. Tel.: (734) 936-9548. Fax: (734) 936-2641. email: mrbenson@umich.edu

Key words: vascular smooth muscle; cell differentiation; transforming growth factor-β; physiologic angiogenesis; transcription factors

Purified normal SMC progenitor cells capable of differentiating into smooth muscle in vitro would provide a system sensitive to gain-of-function effects and would be extremely useful for dissecting the pathways important for smooth muscle differentiation. Such a progenitor cell type—the 10T1/2 cell line—was critically important in the initial identification of MyoD as a master regulator of skeletal muscle differentiation (Olson, 1990). There are a few reports of cells that have the capacity to become smooth muscle in vitro in response to particular stimuli, but these cells either do not acquire a fully differentiated phenotype or only a small fraction of cells actually differentiate (Arciniegas et al., 1992; Drab et al., 1997; Hirschi et al., 1998). In addition, these various cell types have all been subjected to long-term cell culture, increasing the risk of artifactual results.

NCSCs can be purified from rat embryos and have the capacity to differentiate into smooth muscle (Shah et al., 1996; Morrison et al., 1999). This is an excellent system with which to study SMC differentiation—neural crest cells are normal progenitors of vascular smooth muscle, and if stem cells are freshly isolated for each experiment so that time in culture is limited to relatively brief periods, artifactual results due to culture should be minimized. Thus, this system is likely to be much more physiologically relevant than other in vitro SMC differentiation systems.

The work described here examines the effect of calcineurin on NCSCs. Calcineurin is a calcium/calmodulin-activated protein phosphatase, the activation of which results in dephosphorylation of a set of substrates, the best-characterized of which are four NFAT transcription factors, NFATc1–c4 (Rao et al., 1997; Crabtree and Olson, 2002). Phosphorylated, inactive NFATs are localized to the cytoplasm. Dephosphorylation of NFATs results in exposure of a nuclear localization signal and translocation to the nucleus of the cell (Shibasaki et al., 1996; Rao et al., 1997; Crabtree and Olson, 2002). There the NFATs bind to gene regulatory sequences and, through cooperative interactions with other transcription factors, influence gene activity. Nuclear NFATs are rephosphorylated by several kinases, including glycogen synthase kinase (GSK)-3β, resulting in export from the nucleus. Therefore, GSK-3β is an antagonist of calcineurin signaling (Antos et al., 2002; Crabtree and Olson, 2002; Fig. 1, a diagram of the calcineurin signaling pathway).

Here, we describe experiments showing that calcineurin directs NCSCs to a smooth muscle fate, as does the calcineurin-activated transcription factor NFATc1. Two negative regulators of calcineurin signaling, GSK-3β and the calcineurin inhibitor MCIP-1, both decrease smooth muscle differentiation. This work provides strong evidence that calcineurin promotes smooth muscle differentiation in cells of the neural crest lineage. In addition, we present data suggesting that TGF-β1, which also causes NCSCs to differentiate into smooth muscle, appears to do so, at least in part, by activating calcineurin signaling.

Results

### A calcineurin target gene is up-regulated in NCSCs that are differentiating in response to TGF-β1

Rat NCSCs differentiate into smooth muscle in response to TGF- β1 (Shah et al., 1996). In an attempt to identify fac-
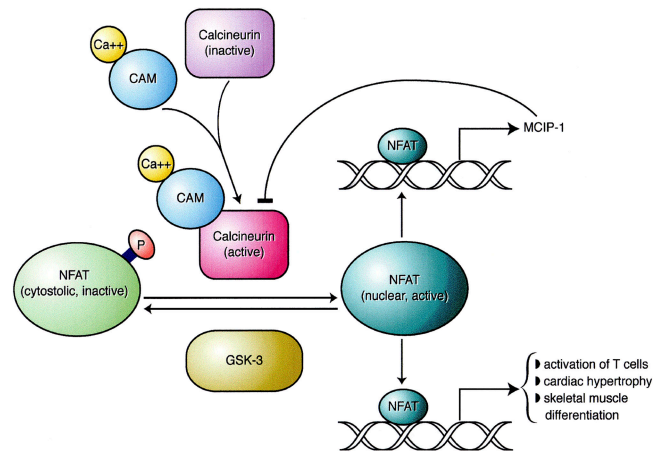
Figure 1. **An outline of the calcineurin signaling pathway.** Calcineurin, a serine/threonine protein phosphatase, is activated by calcium and calmodulin after intracellular calcium levels are elevated for a sustained period of time. Activated calcineurin dephosphorylates several substrates, including the NFAT transcription factors, which then translocate from the cytoplasm to the nucleus and activate a tissue-specific set of genes. In muscle, this set of genes includes MCIP-1, the protein product of which inhibits calcineurin, resulting in a negative feedback loop. Nuclear GSK-3β rephosphorylates nuclear NFATs, so that they are inactivated and exported from the nucleus.

tors that act downstream of TGF-β1 in this process, we constructed a subtracted cDNA library derived from sequences that are up-regulated as NCSCs differentiate into smooth muscle (Moon et al., 2002). One of the up-regulated cDNAs proved to encode MCIP-1, a calcineurin inhibitor (Rothermel et al., 2000; Moon et al., 2002). A virtual
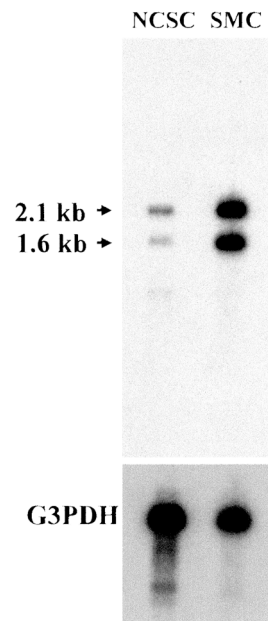
Figure 2. **Virtual Northern blot demonstrates up-regulation of MCIP-1 expression in differentiating NCSC-derived SMCs as compared with undifferentiated NCSCs.** First-strand cDNA from NCSC and SMC was amplified by limited PCR, run on an agarose gel, blotted, and hybridized to an MCIP-1–specific probe. The filter was stripped and hybridized to a G3PDH probe, so that G3PDH serves as an internal control.

Northern blot confirmed up-regulation of MCIP-1 in neural crest-derived differentiating smooth muscle as compared with pluripotent NCSCs (Fig. 2).

Calcineurin activity is chiefly regulated at the posttranslational level (Rao et al., 1997). MCIP-1 gene expression is induced by activated calcineurin (Yang et al., 2000), and so MCIP-1 imposes negative feedback on calcineurin at high levels of calcineurin activity (Fig. 1). Therefore, we reasoned that the increased MCIP-1 mRNA levels in differentiating SMCs might be a surrogate marker for high levels of calcineurin activity in those cells. There is considerable evidence that calcineurin has effects on cardiac muscle hypertrophy, on skeletal muscle fiber type, and on promoting skeletal muscle differentiation (Friday et al., 2000; Rommel et al., 2001; Crabtree and Olson, 2002), so the possibility that calcineurin signaling was activated in differentiating smooth muscle was of great interest.

Activation of the calcineurin signaling pathway increases the SMC fraction of an NCSC population, and inhibition of the calcineurin signaling pathway decreases the SMC fraction of an NCSC population

To evaluate the effect of calcineurin on the differentiation of NCSCs into SMCs, we developed a highly quantitative flow cytometric technique for quickly assessing the fraction of SMCs in a NCSC population. NCSCs were transduced with the pMIG retroviral vector (Van Parijs et al., 1999), which expresses GFP, and were exposed to 20 pM TGF-β1 for 4 d to induce SMC differentiation. These cells were fixed and stained with an antibody directed against smooth muscle α-actin (SMA), a marker for smooth muscle differentiation. An increase in SMC differentiation and a decrease in total cell number was observed for NCSCs treated with TGF-β1, as described previously (Shah et al., 1996; Moon et al., 2002). The fraction of cells that differentiated into NCSCs as measured by high levels of SMA expression was approximately three times that of controls (Fig. 3 A).

We used pMIG to express a constitutively active truncated form of calcineurin in NCSCs. pMIG produces a bicistronic transcript consisting of the inserted sequence as well as sequences encoding GFP, so that transduced cells can be identified by virtue of GFP expression. 6 d after transduction, the cells were fixed and stained with antibody against SMA. We analyzed the cell population by flow cytometry and found that the fraction of SMCs was more than twice as high in cells transduced with the calcineurin construct as compared with cells transduced with vector alone (Fig. 3 B). In addition, we noted that increased GFP correlates with increased SMA expression in the calcineurin-expressing cells. GFP and calcineurin are encoded on the same transcript, and increases in GFP expression should correlate with increased levels of calcineurin expression, as has been demonstrated for other bicistronic transcripts (Collier et al., 1998; Mizuguchi et al., 2000). Therefore, these experimental results suggest that calcineurin promotes smooth muscle differentiation in a dose-dependent manner (Fig. 3 C).

To determine whether or not inhibitors of calcineurin signaling would suppress smooth muscle differentiation, we exploited the fact that NCSCs spontaneously differentiate into smooth muscle at a low frequency. We found that there was a small but consistent inhibition of spontaneous SMC differentiation by MCIP-1 (Fig. 3 B). We also examined the effect of expression of the protein kinase GSK-3β on spontaneous SMC differentiation. GSK-3β has diverse targets, including nuclear NFATs, which it phosphorylates and inactivates. Thus, GSK-3β serves to oppose calcineurin's effects (Antos et al., 2002; Crabtree and Olson, 2002). Expression of constitutively active GSK-3β in NCSCs consistently resulted in a lower rate of spontaneous smooth muscle differentiation (Fig. 3 B), which is consistent with the notion that inhibition of the calcineurin pathway inhibits SMC differentiation. Attempts to use the pharmacological calcineurin inhibitor cyclosporine were unsuccessful because of marked toxicity to NCSCs, even at low concentrations.

Calcineurin acts in an instructive manner to initiate smooth muscle differentiation

The flow cytometry data suggest that calcineurin-expressing cells are more likely to be SMCs than control cells, but it does not tell us anything about the mechanism of that effect. Specifically, flow cytometry does not allow us to discriminate between a selective effect, by which calcineurin is either detrimental to the growth of non-SMC types or promotes proliferation of SMCs, and an instructive effect, by which calcineurin directs progenitor cells to the SMC differentiation pathway. To discriminate between these possibilities, we analyzed the calcineurin effect using clonal analysis.

Freshly isolated NCSCs were transduced with retroviral constructs, and after 24 h the transduced cells were replated at clonal density. Examination 4 h later confirmed that the replated cells were almost exclusively single (91% in three experiments, SD of 7%). 7 d later, the resulting cultures were stained for SMA and each colony was characterized with respect to its SMC content. Each of these colonies has arisen from a single cell, and will therefore consist of cells that are identical in terms of retroviral transduction—all the cells in a colony are either untransduced or transduced, and if transduced, all cells have the same number and chromosomal position of retroviral insertions. The resulting relative homogeneity of phenotype facilitates characterization of each colony and also allows a more rigorous analysis of cell fate decisions. Using clonal analysis, the effect of calcineurin was even more striking than in the flow cytometry experiments (Fig. 4). Clonal analysis of calcineurin-expressing colonies showed a more than eightfold increase in colonies composed entirely of smooth muscle as compared with controls (Fig. 4 B, M). In addition, expression of calcineurin resulted in a more than threefold decrease in the number of colonies that contained no SMCs (Fig. 4 B, U and GU). The ratio of colony number on calcineurin-transduced plates as compared with vector-transduced plates was 1.17, with a SD of 0.04, so there was no evidence for a selective effect that was detrimental to non-SMCs (Fig. 4 B). SMC colonies on calcineurin plates were similar in size to SMC colonies on control plates (unpublished data), so there was no evidence for a selective effect that acts by promoting SMC growth. Our interpretation of this result is that calcineurin acts primarily by instructive means and directs NCSCs toward a smooth muscle fate.
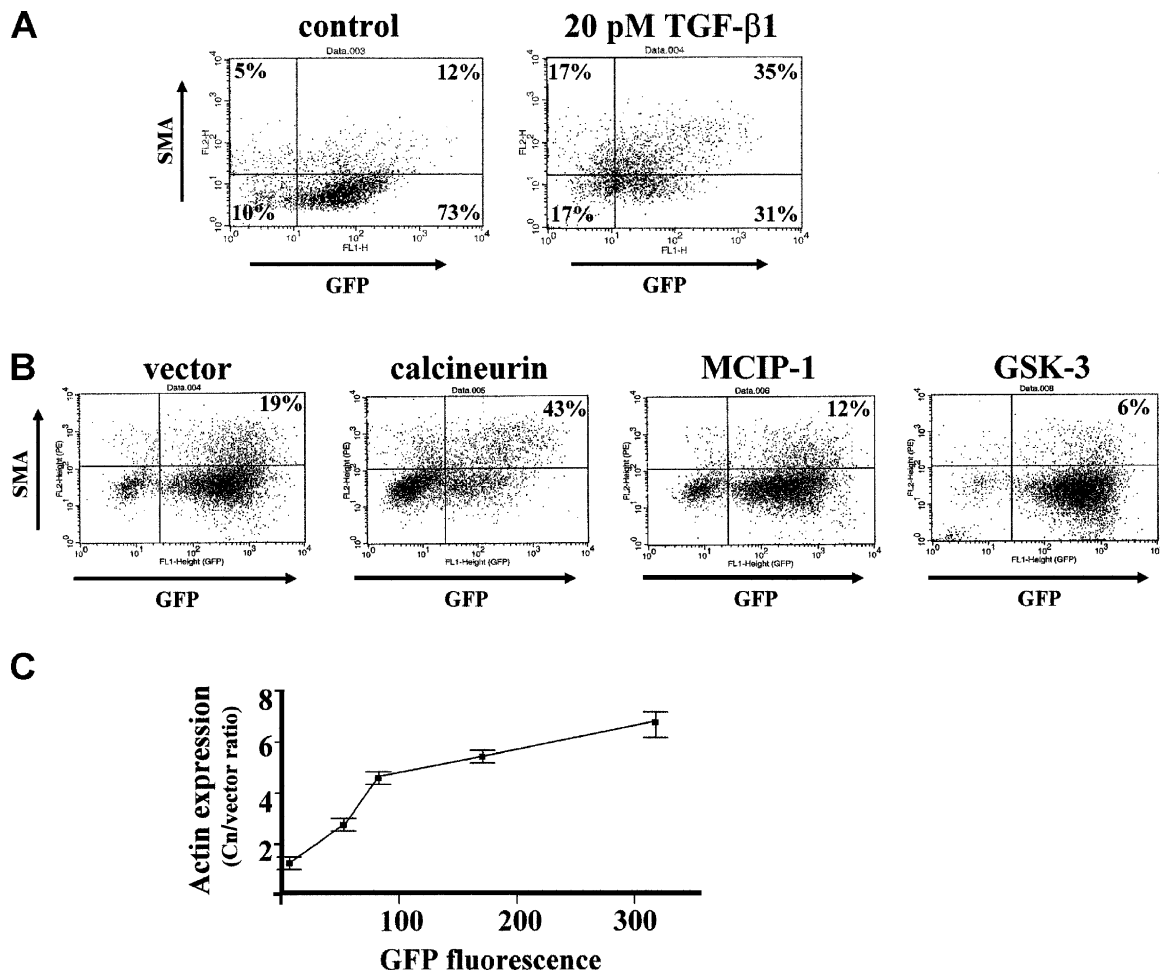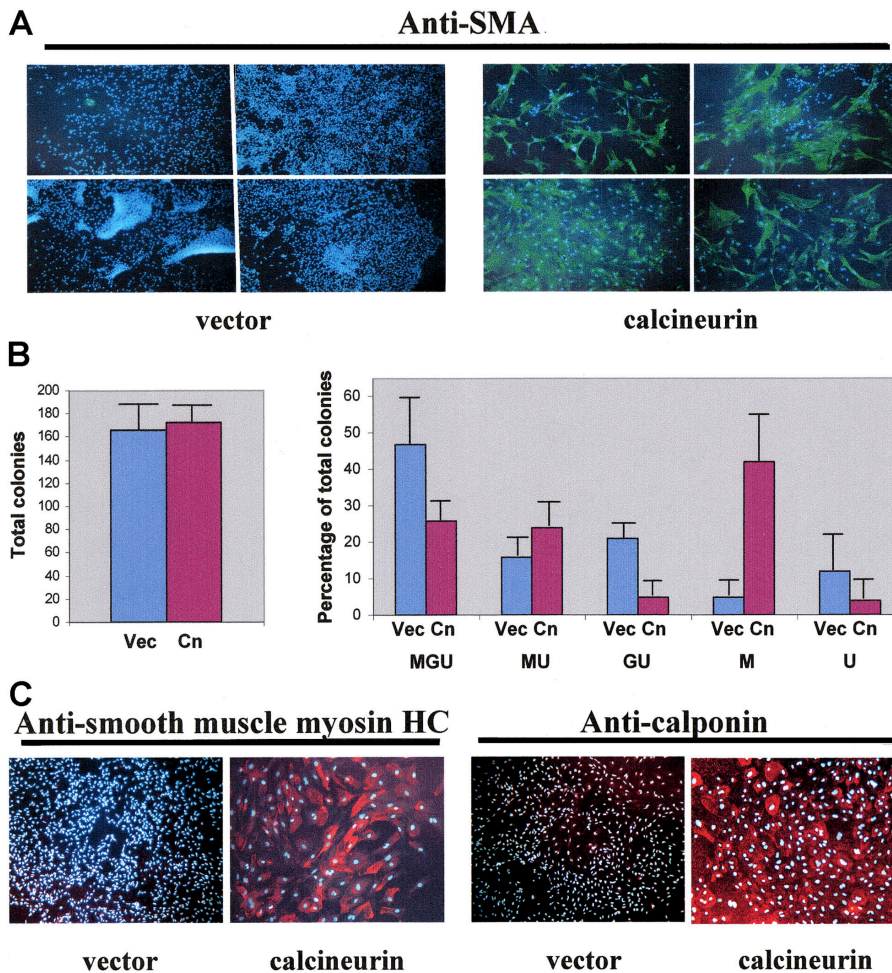
Figure 3. **Calcineurin activation, like TGF-β1 treatment, increases the proportion of smooth muscle in the neural crest cell population.** (A) TGF-mediated smooth muscle differentiation as measured by flow cytometry. Approximately 18,000 NCSCs per well isolated from sciatic nerve were transduced with the pMIG retroviral vector, and 2 d later 20 pM TGF-β1 was added to half of the wells. After four additional days, the cells were fixed and stained with a primary antibody directed against SMA and a PE-conjugated secondary antibody, and PE and GFP fluorescence were determined by flow cytometry. The horizontal and vertical lines on each plot denote the upper boundary of GFP-negative and SMA-negative NCSCs, respectively. Cells to the right of the vertical line are GFP-positive and thus successfully transduced, and cells above the horizontal line are SMA-positive and thus are SMCs. The percentage of cells in each quadrant is indicated. Increased SMC content is evident in wells treated with TGF. (B) Calcineurin expression increases the percentage of SMCs in NCSC cultures, and inhibitors of calcineurin activity decrease the percentage of SMCs in NCSC cultures. NCSCs were transduced the day after isolation, and then grown for 6 d. At that point, they were fixed and stained with a primary antibody directed against SMA and a PE-conjugated secondary antibody, and PE and GFP fluorescence were determined by flow cytometry. The horizontal and vertical lines on each plot denote the upper boundary of GFP-negative and SMA-negative NCSCs, respectively. Cells to the right of the vertical line are GFP-positive and thus successfully transduced, and cells above the horizontal line are SMA-positive and thus are SMCs. The percentage of transduced cells that has differentiated into smooth muscle is indicated in the top right of each plot. Note that the NCSCs in the calcineurin plot that express the highest GFP levels, and therefore presumably the highest calcineurin levels, are almost uniformly smooth muscle. Calcineurin/vector ratio of percentage of SMCs from three independent experiments was 2.4, with a SD of 0.06; for MCIP-1, the mean ratio was 0.6, with a SD of 0.26; and for GSK-3β, the ratio was 0.5, with a SD of 0.15. (C) Increased expression of GFP correlates with increased SMC differentiation. Three independent flow cytometric analyses of calcineurin-transduced cells were evaluated for a correlation between actin expression (i.e., SMC identity) and GFP expression. Because GFP and calcineurin are on a bicistronic message, we expect calcineurin expression to increase in tandem with GFP expression. The level of actin expression at each level of intensity of GFP fluorescence is expressed as fold above a pMIG retroviral vector control ± SD.

To characterize the degree of differentiation that calcineurin is capable of inducing, we stained clonal calcineurin-expressing NCSC colonies with antibodies against smooth muscle myosin heavy chain and against calponin, two proteins that are specific for SMCs. Cells in these colonies express both of these proteins, demonstrating that calcineurin induces a well-differentiated SMC phenotype (Fig. 4 C). We did not detect a qualitative difference between SMCs that arose spontaneously in control cultures and cal-

cineurin-expressing SMCs; both were reactive with these two SMC-specific antibodies. We conclude from this experiment that calcineurin causes NCSCs to differentiate into cells that are smooth muscle as judged by their morphology and by the expression of multiple smooth muscle-specific proteins.

One intriguing aspect of the flow cytometric profile of calcineurin-expressing cells is that a larger fraction of cells are untransduced as compared with flow profiles of MCIP-1

A

Anti-SMA

vector   calcineurin

B

C

Anti-smooth muscle myosin HC   Anti-calponin

vector   calcineurin   vector   calcineurin

Figure 4. **Colonies of calcineurin-transduced NCSCs demonstrate a marked increase in SMC differentiation and assume a highly differentiated phenotype.** NCSCs were transduced 24 h after isolation and replated at clonal density on the following day. They were allowed to grow for 10 d, and then stained with DAPI and the indicated antibodies. (A) Calcineurin- and pMIG-transduced colonies stained with anti-SMA antibody are shown. They were photographed sequentially, in the order that they were encountered on the plate. (B) Colony characterization of calcineurin- and pMIG-transduced colonies. Colonies were stained with cell type–specific antibodies and characterized with respect to their content of undifferentiated (U), smooth muscle (M), or glial (G) content. Cn, calcineurin; Vec, pMIG. Means of three independent experiments plus SD are shown. (C) Calcineurin- or pMIG-transduced SMCs express the highly specific SMC markers calponin and smooth muscle myosin heavy chain, indicating a highly differentiated phenotype. Note that although the degree of magnification may appear to be higher in the photographs of calcineurin-transduced cells, magnification of parallel photomicrographs in A and C is in fact the same. The nuclei of SMCs are larger than those of undifferentiated NCSCs, giving the incorrect impression of different degrees of magnification.

and GSK-3β (Fig. 3 B). The results of other work with the calcineurin-expressing retrovirus led us to doubt that this was simply due to low rates of transduction. Rather, we suspected that this was a result of a slowed mitotic rate in calcineurin-expressing stem cells that have begun to differentiate, so that the proportion of untransduced, undifferentiated cells in the population increased disproportionately over the several days that the cells were cultured. Clonal analysis confirmed this finding; the proportion of GFP-positive colonies on calcineurin plates was equal to that of control plates, demonstrating that transduction rates were equal, and smooth muscle colonies contained many fewer cells than undifferentiated colonies (Fig. 4 A and not depicted), presumably because differentiated SMCs divide more slowly than undifferentiated cells. These data supported our idea that the large number of untransduced cells in flow cytometric studies was due to relative overgrowth of untransduced cells rather than to poor transduction rates.

The calcineurin-activated transcription factor NFATc1 also causes smooth muscle differentiation

The predominant and best studied mechanism by which calcineurin exerts its effects is by dephosphorylating and activating the four calcineurin-regulated NFAT transcription factors. We chose to investigate whether or not calcineurin mediates SMC differentiation via an NFAT-dependent mechanism. We performed RT-PCR experiments to determine which NFAT transcripts are expressed in NCSCs, and found that NFATc1 and NFATc3 are expressed in these cells (unpublished data). Therefore, we constructed a retroviral plasmid with a FLAG-tagged NFATc1 and transduced NCSCs with NFATc1 virus. Fig. 5 shows a photomicrograph of NCSCs expressing NFATc1, and shows that NFATc1 promotes SMC differentiation of NCSCs. In this case, the virus does not coexpress GFP, so although it is not possible to identify transduced cells by virtue of GFP expression, as was done for the experiments shown in Fig. 3, an NFATc1-dependent differentiation effect is clearly evident. This case provides strong evidence that calcineurin causes SMC differentiation in an NFAT-mediated manner.

TGF-β1 activates transcription of the NFATc1 gene

The fact that TGF-β1 activates the expression of MCIP-1, a calcineurin target gene, and the fact that expression of either calcineurin or NFATc1 phenocopies the TGF-β1 effect (i.e., causes smooth muscle differentiation), suggests that TGF-β1 may cause SMC differentiation by activating calcineurin signaling. This is a compelling model because it allows TGF-β1 and calcineurin, two factors known to play a role in SMC differentiation, to be placed in an epistatic relationship in the same differentiation pathway. To further test this model, we quantitated the expression level of another

Figure 5. **Expression of NFATc1 in NCSCs causes smooth muscle differentiation.** (top) Photomicrographs of NCSCs transduced with MSCV vector-derived or FLAG-NFATc1– expressing retrovirus and stained with FLAG antibody. (bottom) Representative example of flow cytometry of NCSCs transduced with MSCV virus (solid black peak) or NFATc1 virus (open peak with gray outline). Cells were fixed and stained for SMA expression 6 d after transduction. NFATc1/vector ratio of percentage of SMCs from three independent experiments was 2.0, with a SD of 0.1.

Vector

NFATc1

Cell number

SMA reactivity
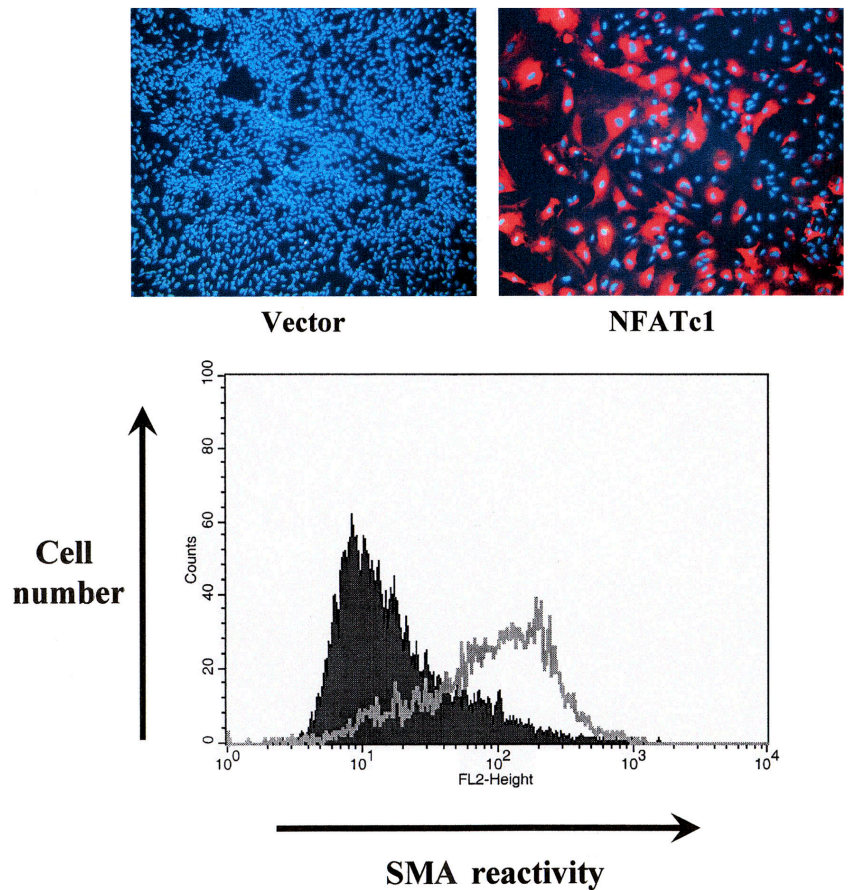
calcineurin/NFAT target gene, NFATc1 itself (Zhou et al., 2002), after TGF-β1 treatment. Using quantitative real-time PCR, we found that TGF-β1 treatment results in a 4.5-fold increase in NFATc1 transcript levels in NCSCs (Fig. 6). This is further evidence that the calcineurin pathway may be activated in TGF-β1–treated NCSCs.

Discussion

Using freshly isolated cells of the neural crest lineage as a smooth muscle differentiation system responsive to gain-of-function effects, we have shown that expression of calcineurin or of the calcineurin-activated transcription factor NFATc1 causes NCSCs to differentiate into smooth muscle and that expression of MCIP-1 or GSK-3β, each of which antagonizes calcineurin signaling, inhibits SMC differentiation in these cells. Clonal analysis indicates that calcineurin exerts its effect on NCSCs by a primarily instructive mechanism rather than a selective mechanism. We found in the clonal analysis experiments that it was necessary to wait 24 h to replate transduced NCSCs because of markedly deleterious effects on cell viability and transduction rate when the cells were replated sooner after transduction. This period of time may allow some selection to take place, but there are several reasons why we think that a significant selective effect is unlikely. First, expression of the retroviral constructs is minimal at 24 h after transduction, and overt differentiation in response to factors expressed from transduced retroviral constructs is not evident until 5–6 d after transduction (un-

published data). Therefore, it seems unlikely that a significant selective effect could be exerted so early after transduction. In addition, comparison of experimental plates with controls shows no evidence of a selective effect. Therefore, we conclude that the effect of calcineurin on NCSCs is primarily, and perhaps exclusively, instructive.

The fact that calcineurin is sufficient to cause SMC differentiation shows that it is capable of initiating the smooth muscle differentiation program. This is a conclusion that could not have been made from whole animal loss-of-function experiments alone and demonstrates the value of a physiologically relevant in vitro differentiation system.

The observation that calcineurin plays a role in smooth muscle differentiation is not entirely surprising because the importance of calcineurin signaling to cardiac and skeletal muscle has already been established. Several groups have shown that calcineurin is capable of causing cardiac hypertrophy, skeletal muscle fiber type switching, and skeletal muscle differentiation (Chin et al., 1998; Friday et al., 2000; Kegley et al., 2001; Crabtree and Olson, 2002). In addition, the phenotypes of mice deficient for calcineurin pathway function are consistent with a role for calcineurin signaling in SMC differentiation (Graef et al., 2001). Mice with a disrupted calcineurin regulatory subunit (CnB) gene and mice with two disrupted NFAT genes (NFATc3 and NFATc4) have noticeable abnormalities in vSMC differentiation. In these mice, aortic SMCs are in many cases not tightly associated with the vessel wall. In CnB knockout mice, immunoreactivity of vSMCs with smooth muscle-specific antibodies is
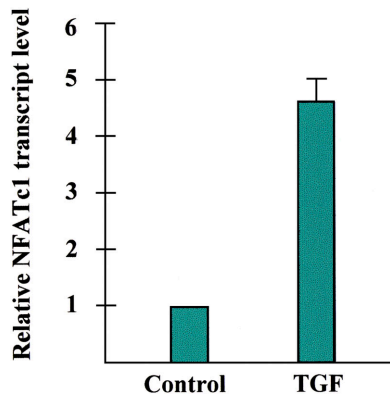
Figure 6. **NFATc1 transcript levels increase in TGF-treated NCSCs.** NCSCs were treated with TGF-β1 for 24–48 h, and then total RNA was isolated and quantitative real-time PCR was performed using NFATc1-specific primers. Both GAPDH and β-actin transcripts were also quantitated, and levels of NFATc1 transcript were normalized to either GAPDH or β-actin. The result was virtually identical using either GAPDH or β-actin to normalize (mean of 4.6, SD of 0.4 or mean of 4.5, SD of 0.5, respectively, three independent experiments).

dramatically decreased. The authors speculate that there is no intrinsic, cell-autonomous defect in SMC differentiation because somitic vessel SMCs look normal, but this finding could also be explained by differing sensitivities of SMCs from different lineages to defects in calcineurin signaling. Vertebrate vascular smooth muscle is derived from multiple cell types. SMCs in the proximal aorta, aortic arch, and pulmonary trunk are derived from neural crest, whereas smooth muscle in the descending aorta is derived from mesoderm and probably also from transdifferentiated endothelium (Gittenberger-de Groot et al., 1999; Jiang et al., 2000). Smooth muscle in the coronary arteries is derived from epicardium (Gittenberger-de Groot et al., 1999). SMCs from different progenitor cell types have different properties, and thus vascular smooth muscle in one part of the animal is different from vascular smooth muscle in another part (Topouzis and Majesky, 1996). Our experiments, which show that calcineurin initiates SMC differentiation in purified NCSCs, support a model of smooth muscle differentiation wherein the calcineurin requirement is cell-autonomous in nature. A tenable alternative interpretation of the whole animal loss-of-function experiments is that the calcineurin requirement is cell-autonomous but that different progenitor cell types have differing sensitivities to loss of calcineurin signaling.

Loss-of-function mutations in the gene encoding the MEF2C transcription factor also result in poor vSMC differentiation (Lin et al., 1998). The fact that MEF family members are activated by calcineurin in cardiac and skeletal muscle (Olson and Williams, 2000; Zhang et al., 2002) and are known to interact with NFAT transcription factors at target promoters constitutes further evidence of a role for calcineurin in smooth muscle differentiation. It will be important to examine potential relationships between calcineurin and MEF2C in smooth muscle differentiation. Similarly, potential relationships between calcineurin and other factors likely to be involved in smooth muscle differentiation, like TGF-β1, GATA-6, and SRF (Folkman and D'Amore, 1996; Parmacek, 2001), will be of great interest.

The mechanisms of NFAT-mediated calcineurin signaling were elucidated primarily in immune cells (Rao et al., 1997; Crabtree and Olson, 2002). Interestingly, the actions of calcineurin in muscle and in T cells are mediated in strikingly similar ways (Crabtree and Olson, 2002). For example, calcineurin signaling in lymphocytes seems to require a cooperative interaction between NFATs and other transcription factors, like members of the MEF family and AP-1 (Liu et al., 1997; Rao et al., 1997). Calcineurin-mediated skeletal muscle fiber type switching also seems to act via an association between NFAT and MEF transcription factors (Calvo et al., 1999; Wu et al., 2000). This finding suggests that the mechanisms of NFAT-mediated calcineurin signaling are likely to be conserved, in general outline and perhaps in significant detail, in NCSCs.

Activation of the calcineurin signaling pathway in response to TGF-β1 has not been previously described, yet our experiments suggest that it occurs in NCSCs. We see up-regulation of MCIP-1 and NFATc1, both of which are calcineurin target genes, in response to TGF-β1. In addition, expression of either calcineurin or NFATc1 mimics the effects of TGF-β1 on NCSCs. This suggests an epistatic relationship between TGF-β1 and the calcineurin pathway in smooth muscle differentiation, a relationship in which TGF-β1 activates calcineurin signaling to initiate the differentiation program. The discovery of this potential relationship between the TGF-β1 and calcineurin pathways highlights the strengths of the NCSC system as a tool to study SMC differentiation and demonstrates how NCSCs can be used to better define relationships between various factors known to be important for SMC differentiation.

TGF-β1 might activate calcineurin by any one of a variety of mechanisms. It has been demonstrated that in some cell types, TGF-β1 is capable of increasing intracellular calcium to levels adequate for calcineurin activation (Alevizopoulos et al., 1997). Therefore, direct activation of calcineurin by TGF-β1 is one plausible mechanism. Activation of calcineurin would lead to dephosphorylation and activation of NFATs, and then to transcriptional activation of NFAT target genes, including MCIP-1 and NFATc1. Other possible mechanisms include direct activation of genes encoding more downstream components of the calcineurin signaling pathway, such as NFATc1, by TGF-β1, in a Smad-dependent or -independent manner.

The convincing demonstration that activation of the calcineurin pathway by TGF-β1 is necessary for TGF-β1–induced smooth muscle differentiation, and a precise definition of the mechanism by which TGF-β1 activates the calcineurin signaling pathway, would be of great interest. Experiments to accomplish these aims are ongoing in our laboratory.

Materials and methods

NCSC isolation

NCSC isolation has been described in detail previously (Morrison et al., 1999; Bixby et al., 2002; Kruger et al., 2002; Moon et al., 2002). In brief, rat E14.5 sciatic nerves or gut (stomach and intestine) were dissected. Disaggregated sciatic nerve cells were stained with antibodies to p75 and P0, and cells with high p75 and low P0 were isolated by FACS®. Disaggregated gut cells were stained with antibodies to p75 and α-4, and cells with

high p75 and high α-4 were isolated by FACS®. NCSCs were cultured at all times in low oxygen chambers (1% oxygen, 5% CO_2). Gut and sciatic nerve stem cells responded in an identical manner to transduction with calcineurin, MCIP-1, and GSK-3β.

Generation of a subtracted library specific for differentiating smooth muscle

This generation was described previously (Moon et al., 2002). In brief, sciatic nerve stem cells were treated with 20 pM TGF-β1 for 24 h, and then mRNA was isolated and cDNA made. This cDNA library was subtracted with cDNA from pluripotent NCSCs that had not been treated with TGF-β1.

Virtual Northern blot of MCIP-1

Virtual Northern blot of MCIP-1 was described previously (Moon et al., 2002). Full-length first-strand cDNA from untreated, pluripotent NCSCs or from NCSCs treated with TGF-β1 for 24 h was amplified for a limited number of cycles using the Advantage system (CLONTECH Laboratories, Inc.), which should result in proportional amplification. The cDNA was run out on an agarose gel, blotted, and hybridized to an MCIP-1–specific probe using standard procedures. Blots were stripped and reprobed with GAPDH as an internal control.

Cloning of constitutively active calcineurin, NFATc1, GSK-3β, and MCIP-1 into the pMIG vector

High fidelity PCR was used to obtain cDNAs encoding MCIP-1, a truncated calcineurin coding sequence (1–398 aa), and S9A GSK-3β from rat cDNA. The calcineurin and GSK proteins produced by these cDNAs are constitutively active (O'Keefe et al., 1992; Ohteki et al., 2000). All PCR products were sequenced to ensure that no PCR-induced mutations had occurred and were cloned into pMIG using standard techniques. A cDNA encoding human NFATc1 was subcloned from a construct provided by G. Crabtree (Stanford University, Stanford, CA).

Production of retrovirus and transduction of NCSCs

The BOSC packaging cell line (Pear et al., 1993; Pear, 1996) was transfected with pMIG constructs using standard calcium phosphate techniques. 250 ng of the pHCMV-G plasmid (a gift from N. Hopkins, Massachusetts Institute of Technology, Cambridge, MA), which encodes vesicular stomatitis virus glycoprotein, was cotransfected with each pMIG construct. Media were changed the next day, and 18 h later the media were taken off and used as vesicular stomatitis virus glycoprotein–pseudotyped viral stock (Hopkins, 1993). NCSCs were transduced by removing half the medium in each well and replacing it with viral stock. Polybrene was also added, to a final concentration of 1 μg/ml. Cells were incubated for 4 h, whereupon the media were replaced.

Flow cytometry of transduced NCSCs

NCSCs that had been transduced with retrovirus were fixed with the Cytofix/Cytoperm kit (BD Biosciences). After fixation, cells were stained with an antibody to SMA (anti-SMA; A2547; Sigma-Aldrich) and a phycoerythrin (PE)-conjugated secondary antibody (115-116-146; Jackson ImmunoResearch Laboratories). 10,000 cells from each sample were assessed for GFP and PE fluorescence by a FACScan flow cytometer (Becton Dickinson). Mock-transduced cells were used as a GFP-negative control, and cells stained with nonspecific isotype-matched IgG (554126; BD Biosciences) in place of anti-SMA were used as an actin-negative control.

Clonal analysis

300–400 NCSCs were sorted to each well of a 12-well plate, and were transduced on the following day. The day after transduction, the cells were removed with trypsin and immediately replated. Each well of a 12-well plate was replated to 6 wells of a 6-well plate. The cells were grown for 6–10 d, whereupon they were inspected by fluorescence microscopy and GFP-positive colonies were identified. In most experiments, all or very nearly all colonies were GFP positive, indicating that they had been effectively transduced. After confirming successful transduction, the plates were stained with anti-SMA, anti-glial fibrillary acidic protein (anti-GFAP; G3893; Sigma-Aldrich), a PE-conjugated antibody reactive with anti-GFAP (Southern Biotechnology Associates 1070–09), a FITC-conjugated antibody reactive against anti-SMA (1080-02; Southern Biotechnology Associates, Inc.), and DAPI. Colonies were visualized on an epifluorescence microscope (model Diaphot 300; Nikon) and photographed with a 35-mm camera (model FX-35DX; Nikon), using NPZ 800 ISO film (Fuji), with the camera set at 1600 ISO. Photographs were scanned into Photoshop using a scanner (model Expression 1680; Epson).

Immunostaining of transduced colonies

Cells were fixed with acid/ethanol (Kruger et al., 2002) and stained with anti-SMA, anti-GFAP, and DAPI as described in the preceding section. In the experiment shown in Fig. 4 C, cells were stained with antibody against smooth muscle myosin heavy chain (M7786; Sigma-Aldrich) or anticalponin (C2687; Sigma-Aldrich). The same secondary antibody (1070-09; Southern Biotechnology Associates, Inc.) was used for both of these primary antibodies. Colonies were examined, characterized, and photographed under a fluorescence microscope as described for the clonal analysis experiments.

Quantitative real-time PCR

PCR primers that amplified a fragment of 80–160 bp were designed and were shown to target a unique sequence by using BLAST searches of the rat genome. 500–1,500 NCSCs (from gut or sciatic nerve) were sorted to each well of a 12-well plate and grown for 5–6 d, at which time TGF-β1 was added to a final concentration of 20 pM. 24–48 h later, total RNA was isolated using the RNAqueous-Micro kit (Ambion). First-strand cDNA was made from total RNA by random priming using the SuperScript First Strand Synthesis System (GIBCO BRL), yielding a final volume of 20 μl cDNA. Quantitative real-time PCR reactions were performed on a LightCycler (Roche), using FastStart DNA Master SYBR Green I reagents (Roche). A 1-μl volume of cDNA (either undiluted or diluted 1:5 with water) was used in each quantitative real-time PCR reaction. Cycle number for each reaction was determined by taking the crossing point of a tangent to the log-linear portion of the curve of cycle number versus fluorescence. Relative amounts of cDNA (target compared with internal control) were determined using an estimated efficiency of 1.8-fold amplification per cycle. Melting curve analysis was performed for each reaction to rule out primer dimer formation and to ensure that a unique product was generated.

References

Alevizopoulos, A., Y. Dusserre, U. Ruegg, and N. Mermod. 1997. Regulation of the transforming growth factor beta-responsive transcription factor CTF-1 by calcineurin and calcium/calmodulin-dependent protein kinase IV. *J. Biol. Chem.* 272:23597–23605.

Antos, C.L., T.A. McKinsey, N. Frey, W. Kutschke, J. McAnally, J.M. Shelton, J.A. Richardson, J.A. Hill, and E.N. Olson. 2002. Activated glycogen synthase-3 beta suppresses cardiac hypertrophy in vivo. *Proc. Natl. Acad. Sci. USA.* 99:907–912.

Arciniegas, E., A.B. Sutton, T.D. Allen, and A.M. Schor. 1992. Transforming growth factor beta 1 promotes the differentiation of endothelial cells into smooth muscle-like cells in vitro. *J. Cell Sci.* 103:521–529.

Bixby, S., G.M. Kruger, J.T. Mosher, N.M. Joseph, and S.J. Morrison. 2002. Cell-intrinsic differences between stem cells from different regions of the peripheral nervous system regulate the generation of neural diversity. *Neuron.* 35:643–656.

Calvo, S., P. Venepally, J. Cheng, and A. Buonanno. 1999. Fiber-type-specific transcription of the troponin I slow gene is regulated by multiple elements. *Mol. Cell. Biol.* 19:515–525.

Carmeliet, P. 2000. Mechanisms of angiogenesis and arteriogenesis. *Nat. Med.* 6:389–395.

Chang, D.F., N.S. Belaguli, D. Iyer, W.B. Roberts, S.P. Wu, X.R. Dong, J.G. Marx, M.S. Moore, M.C. Beckerle, M.W. Majesky, and R.J. Schwartz. 2003. Cysteine-rich LIM-only proteins CRP1 and CRP2 are potent smooth muscle differentiation cofactors. *Dev. Cell.* 4:107–118.

Chin, E.R., E.N. Olson, J.A. Richardson, Q. Yang, C. Humphries, J.M. Shelton, H. Wu, W. Zhu, R. Bassel-Duby, and R.S. Williams. 1998. A calcineurin-

dependent transcriptional pathway controls skeletal muscle fiber type. *Genes Dev.* 12:2499–2509.

Collier, A.J., S. Tang, and R.M. Elliott. 1998. Translation efficiencies of the 5′ untranslated region from representatives of the six major genotypes of hepatitis C virus using a novel bicistronic reporter assay system. *J. Gen. Virol.* 79:2359–2366.

Crabtree, G.R., and E.N. Olson. 2002. NFAT signaling: choreographing the social lives of cells. *Cell.* 109(Suppl):S67–S79.

Drab, M., H. Haller, R. Bychkov, B. Erdmann, C. Lindschau, H. Haase, I. Morano, F.C. Luft, and A.M. Wobus. 1997. From totipotent embryonic stem cells to spontaneously contracting smooth muscle cells: a retinoic acid and db-cAMP in vitro differentiation model. *FASEB J.* 11:905–915.

Du, K.L., H.S. Ip, J. Li, M. Chen, F. Dandre, W. Yu, M.M. Lu, G.K. Owens, and M.S. Parmacek. 2003. Myocardin is a critical serum response factor cofactor in the transcriptional program regulating smooth muscle cell differentiation. *Mol. Cell. Biol.* 23:2425–2437.

Folkman, J., and P.A. D'Amore. 1996. Blood vessel formation: what is its molecular basis? *Cell.* 87:1153–1155.

Friday, B.B., V. Horsley, and G.K. Pavlath. 2000. Calcineurin activity is required for the initiation of skeletal muscle differentiation. *J. Cell Biol.* 149:657–666.

Gittenberger-de Groot, A.C., M.C. DeRuiter, M. Bergwerff, and R.E. Poelmann. 1999. Smooth muscle cell origin and its relation to heterogeneity in development and disease. *Arterioscler. Thromb. Vasc. Biol.* 19:1589–1594.

Graef, I.A., F. Chen, L. Chen, A. Kuo, and G.R. Crabtree. 2001. Signals transduced by Ca^{2+}/calcineurin and NFATc3/c4 pattern the developing vasculature. *Cell.* 105:863–875.

Hirschi, K., S. Rohovsky, and P. D'Amore. 1998. PDGF, TGFβ, and heterotypic cell–cell interactions mediate endothelial cell-induced recruitment of 10T1/2 cells and their differentiation to a smooth muscle fate. *J. Cell Biol.* 141:805–814.

Hopkins, N. 1993. High titers of retrovirus (vesicular stomatitis virus) pseudotypes, at last. *Proc. Natl. Acad. Sci. USA.* 90:8759–8760.

Jiang, X., D.H. Rowitch, P. Soriano, A.P. McMahon, and H.M. Sucov. 2000. Fate of the mammalian cardiac neural crest. *Development.* 127:1607–1616.

Kegley, K.M., J. Gephart, G.L. Warren, and G.K. Pavlath. 2001. Altered primary myogenesis in NFATC3$^{-/-}$ mice leads to decreased muscle size in the adult. *Dev. Biol.* 232:115–126.

Kruger, G.M., J.T. Mosher, S. Bixby, N. Joseph, T. Iwashita, and S.J. Morrison. 2002. Neural crest stem cells persist in the adult gut but undergo changes in self-renewal, neuronal subtype potential, and factor responsiveness. *Neuron.* 35:657–669.

Li, D.Y., L.K. Sorensen, B.S. Brooke, L.D. Urness, E.C. Davis, D.G. Taylor, B.B. Boak, and D.P. Wendel. 1999. Defective angiogenesis in mice lacking endoglin. *Science.* 284:1534–1537.

Lin, Q., J. Lu, H. Yanagisawa, R. Webb, G.E. Lyons, J.A. Richardson, and E.N. Olson. 1998. Requirement of the MADS-box transcription factor MEF2C for vascular development. *Development.* 125:4565–4574.

Liu, S., P. Liu, A. Borras, T. Chatila, and S.H. Speck. 1997. Cyclosporin A-sensitive induction of the Epstein-Barr virus lytic switch is mediated via a novel pathway involving a MEF2 family member. *EMBO J.* 16:143–153.

Mano, T., Z. Luo, S.L. Malendowicz, T. Evans, and K. Walsh. 1999. Reversal of GATA-6 downregulation promotes smooth muscle differentiation and inhibits intimal hyperplasia in balloon-injured rat carotid artery. *Circ. Res.* 84:647–654.

Mizuguchi, H., Z. Xu, A. Ishii-Watabe, E. Uchida, and T. Hayakawa. 2000. IRES-dependent second gene expression is significantly lower than cap-dependent first gene expression in a bicistronic vector. *Mol. Ther.* 1:376–382.

Moon, E.S., J.L. Ray, R.L. Leach, and M.R. Benson. 2002. A set of genes activated in differentiating smooth muscle is also activated in smooth muscle from injured arteries or atherosclerotic lesions. *Gene Function & Disease.* 3:77–86.

Morrisey, E.E., H.S. Ip, M.M. Lu, and M.S. Parmacek. 1996. GATA-6: a zinc finger transcription factor that is expressed in multiple cell lineages derived from lateral mesoderm. *Dev. Biol.* 177:309–322.

Morrison, S.J., P.M. White, C. Zock, and D.J. Anderson. 1999. Prospective identification, isolation by flow cytometry, and in vivo self-renewal of multipotent mammalian neural crest stem cells. *Cell.* 96:737–749.

Oh, S.P., T. Seki, K.A. Goss, T. Imamura, Y. Yi, P.K. Donahoe, L. Li, K. Miyazono, P. ten Dijke, S. Kim, and E. Li. 2000. Activin receptor-like kinase 1 modulates transforming growth factor-beta 1 signaling in the regulation of angiogenesis. *Proc. Natl. Acad. Sci. USA.* 97:2626–2631.

Ohteki, T., M. Parsons, A. Zakarian, R.G. Jones, L.T. Nguyen, J.R. Woodgett, and P.S. Ohashi. 2000. Negative regulation of T cell proliferation and interleukin 2 production by the serine threonine kinase GSK-3. *J. Exp. Med.* 192:99–104.

O'Keefe, S.J., J. Tamura, R.L. Kincaid, M.J. Tocci, and E.A. O'Neill. 1992. FK-506- and CsA-sensitive activation of the interleukin-2 promoter by calcineurin. *Nature.* 357:692–694.

Olson, E.N. 1990. MyoD family: a paradigm for development? *Genes Dev.* 4:1454–1461.

Olson, E.N., and R.S. Williams. 2000. Remodeling muscles with calcineurin. *Bioessays.* 22:510–519.

Parmacek, M.S. 2001. Transcriptional programs regulating vascular smooth muscle cell development and differentiation. *Curr. Top. Dev. Biol.* 51:69–89.

Pear, W. 1996. Transient transfection methods for preparation of high-titer retroviral supernatants. *In* Current Protocols in Molecular Biology. Vol. 2. F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl, editors. John Wiley & Sons, Inc., New York. 9.11.

Pear, W.S., G.P. Nolan, M.L. Scott, and D. Baltimore. 1993. Production of high-titer helper-free retroviruses by transient transfection. *Proc. Natl. Acad. Sci. USA.* 90:8392–8396.

Perrella, M.A., M.K. Jain, and M.E. Lee. 1998. Role of TGF-beta in vascular development and vascular reactivity. *Miner. Electrolyte Metab.* 24:136–143.

Rao, A., C. Luo, and P.G. Hogan. 1997. Transcription factors of the NFAT family: regulation and function. *Annu. Rev. Immunol.* 15:707–747.

Rommel, C., S.C. Bodine, B.A. Clarke, R. Rossman, L. Nunez, T.N. Stitt, G.D. Yancopoulos, and D.J. Glass. 2001. Mediation of IGF-1-induced skeletal myotube hypertrophy by PI(3)K/Akt/mTOR and PI(3)K/Akt/GSK3 pathways. *Nat. Cell Biol.* 3:1009–1013.

Rothermel, B., R.B. Vega, J. Yang, H. Wu, R. Bassel-Duby, and R.S. Williams. 2000. A protein encoded within the Down syndrome critical region is enriched in striated muscles and inhibits calcineurin signaling. *J. Biol. Chem.* 275:8719–8725.

Shah, N.M., A.K. Groves, and D.J. Anderson. 1996. Alternative neural crest cell fates are instructively promoted by TGFbeta superfamily members. *Cell.* 85:331–343.

Shibasaki, F., E.R. Price, D. Milan, and F. McKeon. 1996. Role of kinases and the phosphatase calcineurin in the nuclear shuttling of transcription factor NF-AT4. *Nature.* 382:370–373.

Topouzis, S., and M.W. Majesky. 1996. Smooth muscle lineage diversity in the chick embryo. Two types of aortic smooth muscle cell differ in growth and receptor-mediated transcriptional responses to transforming growth factor-beta. *Dev. Biol.* 178:430–445.

Van Parijs, L., Y. Refaeli, J.D. Lord, B.H. Nelson, A.K. Abbas, and D. Baltimore. 1999. Uncoupling IL-2 signals that regulate T cell proliferation, survival, and Fas-mediated activation-induced cell death. *Immunity.* 11:281–288.

Wang, D., P.S. Chang, Z. Wang, L. Sutherland, J.A. Richardson, E. Small, P.A. Krieg, and E.N. Olson. 2001. Activation of cardiac gene expression by myocardin, a transcriptional cofactor for serum response factor. *Cell.* 105:851–862.

Wu, H., F.J. Naya, T.A. McKinsey, B. Mercer, J.M. Shelton, E.R. Chin, A.R. Simard, R.N. Michel, R. Bassel-Duby, E.N. Olson, and R.S. Williams. 2000. MEF2 responds to multiple calcium-regulated signals in the control of skeletal muscle fiber type. *EMBO J.* 19:1963–1973.

Yamagishi, H., E.N. Olson, and D. Srivastava. 2000. The basic helix-loop-helix transcription factor, dHAND, is required for vascular development. *J. Clin. Invest.* 105:261–270.

Yang, J., B. Rothermel, R.B. Vega, N. Frey, T.A. McKinsey, E.N. Olson, R. Bassel-Duby, and R.S. Williams. 2000. Independent signals control expression of the calcineurin inhibitory proteins MCIP1 and MCIP2 in striated muscles. *Circ. Res.* 87:E61–E68.

Zhang, C.L., T.A. McKinsey, S. Chang, C.L. Antos, J.A. Hill, and E.N. Olson. 2002. Class II histone deacetylases act as signal-responsive repressors of cardiac hypertrophy. *Cell.* 110:479–488.

Zhou, B., R.Q. Cron, B. Wu, A. Genin, Z. Wang, S. Liu, P. Robson, and H.S. Baldwin. 2002. Regulation of the murine Nfatc1 gene by NFATc2. *J. Biol. Chem.* 277:10704–10711.