RhoA/ROCK Signaling Regulates Chondrogenesis in a Context-dependent Manner*

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The development of the cartilage template that precedes endochondral bone formation requires the condensation of mesenchymal cells and their subsequent differentiation to the chondrocytic lineage. We have previously shown that inhibition of the RhoA/ROCK signaling pathway or actin dynamics enhances Sox9 mRNA expression, increases glycosaminoglycan production, and transforms cell shape to a spherical, chondrocyte-like morphology. However, we demonstrate here that in three-dimensional micromass cultures of mesenchymal cells, increased expression of Sox9 in response to these manipulations is not sufficient to induce the expression of established Sox9 target genes. This is illustrated by a decrease in the transcript levels of collagen II and aggrecan as well as reduced activity of a Sox9-responsive reporter gene in response to ROCK inhibition and cytochalasin D. We also demonstrate a decrease in mRNA levels of the transcriptional co-activators L-Sox5 and Sox6 upon ROCK inhibition and cytochalasin D. The decrease in Sox9 activity is likely partially due to reduced L-Sox5 and Sox6 levels but also to a delay in Sox9 phosphorylation following ROCK inhibition. In contrast, inhibition of the RhoA/ROCK pathway and cytochalasin D treatment in monolayer culture results in the enhancement of a number of markers of chondrogenesis such as Sox9 activity and collagen II and aggrecan transcripts levels. These data demonstrate that the effects of RhoA/ROCK signaling and actin polymerization inhibitors on chondrogenic gene expression are dependent on the cellular context.

Endochondral ossification is a developmental process that forms the majority of the mammalian skeleton, as reviewed in Ref. 1. Beginning with condensations of mesenchymal cells and subsequent differentiation to the chondrocytic lineage, precisely shaped cartilage templates are formed (2). These templates are composed of proliferating and differentiating chondrocytes as well as vast amounts of extracellular matrix produced by chondrocytes (3). The commitment of cells to the chondrocytic lineage is marked by change in cell shape (from a spread, fibroblastic-like morphology to a spherical morphology), expression of the essential transcription factor Sox9 (4) and the matrix markers collagen II (5) and aggrecan (6), and increased glycosaminoglycan production (1).

Although the importance of cell shape is recognized (7), molecular mechanisms controlling these processes have not been studied extensively. The RhoA/ROCK signaling pathway is an excellent candidate as its role as a master regulator of the actin cytoskeleton has been well documented (8–10). In addition, RhoA signaling controls cell cycle progression (11), differentiation (12), and apoptosis in other cell types (13). We recently demonstrated that inhibition of the RhoA/ROCK pathway by the pharmacological inhibitor Y27632 induced spherical cell morphology, increased the transcription of the chondrogenic transcription factor Sox9, and enhanced glycosaminoglycan accumulation (14). We also demonstrated that RhoA overexpression in the cell line ATDC5 had the opposite effect on these markers of chondrogenesis. Additionally, we showed that jasplakinolide and cytochalasin D, pharmacological modifiers of cytoskeletal dynamics, also played a central role in regulating Sox9 transcript levels (14).

As mentioned above, the commitment of mesenchymal cells to the chondrocytic lineage is marked by the expression of the transcription factor, Sox9 (4). Sox9 is required for cartilage formation (15) and directly activates transcription of the collagen II (16) and aggrecan (17) genes. It has been more recently recognized that the transcription factors L-Sox5 and Sox6 are also required for proper cartilage formation (18). These factors are co-expressed with Sox9 (19) and allow for maximal activity of Sox9 (20). L-Sox5 and Sox6 are highly homologous to each other and contain coiled-coil domains, allowing for homo- and heterodimerization (19). Heterodimerization of L-Sox5 to Sox6 enhances binding to target DNA and, with co-operative binding of Sox9, enhances transcription of several essential cartilage extracellular matrix genes, such as collagen II (19) and aggrecan (18) genes. The activity of the Sox trio is also enhanced by the specific phosphorylation of Sox9 by cAMP-dependent protein kinase on the serine residue, 181 (21).

Although our previous results clearly demonstrated stimulation of Sox9 expression by inhibition of ROCK or actin dynamics, we had not analyzed the effects of these treatments of downstream markers of chondrogenesis (14). We show that although Sox9 transcript levels are increased in micromass cultures treated with the ROCK inhibitor Y27632 or cytochalasin D, mRNA levels of the Sox9 target genes collagen II and aggrecan are reduced under the same conditions. This corresponds to reduced levels of Sox5 and Sox6 transcripts. Moreover, we show that there is a deregulation of the temporal phosphorylation patterns of Sox9 in Y27632-treated micromass cultures. Stabilization of actin by jasplakinolide increased transcription of Sox9 but did not adversely affect the activity of Sox9 in micromass cultures. Furthermore, we show that Sox9 activity is not negatively affected in other culture models, such as the ATDC5 cell line and high density monolayer culture of growth plate chondrocytes, suggesting that the effects of the RhoA/ROCK pathway on chondrogenic gene expression are context-dependent.

EXPERIMENTAL PROCEDURES

Materials

CD1 mice were timed impregnated and were purchased from Charles River Laboratories. All cell culture media components were purchased from Invitrogen or Sigma unless stated otherwise. All inhibitors were purchased from Calbiochem or Sigma. All other reagents were of ana
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Primary chondrocytes isolated from embryonic day 15.5 growth plates were isolated as described above and plated at a density of 100,000 cells/well in a 24-well dish. A suspension of 1:1 FuGENE 6 and DNA (as described above) was added to the medium for 4 h. After this time, the media were changed, and inhibitors were added. Cells were isolated in lysate buffer after 2 days in culture and assayed as described above.

Statistical Analysis—Data collected from the RhoA activity assay are an average of three independent experiments of samples analyzed in triplicate. The mean values were determined by averaging triplicate wells and subtracting the negative control (lysis buffer only) from all experimental values. The data were normalized to day 3 of culture. Data collected from real-time RT3-PCR are an average of three independent experiments, each analyzed in triplicate. Means were quantified relative to GAPDH, and then data were normalized to day 1 of control-treated RNA per trial. Data collected from the luciferase assays are an average of three independent experiments, each analyzed in triplicate. Relative light units were quantified by normalizing target luciferase (firefly) relative to the CMV control (Renilla). Statistical significance was determined by a one-way (luciferase data) or two-way (real-time RT-PCR analysis) analysis of variance with Bonferroni post significance was determined by size as described (14). Protein was also isolated from days 3, 6, and 9 of culture as described above from the cell line ATDC5 treated with MeSO vehicle or 10 μM Y27632. Blotted membranes were incubated with one μg/ml primary antibody against pS181 Sox9 (Biosource) or β-actin and incubated overnight at 4 °C followed by 5000× dilution of the secondary antibody for 1 h at 4 °C. Signal was detected using ECL Western blotting detection reagents (Amersham Biosciences) according to the manufacturer’s protocol and visualized on the ChemiImager 5500 (AlphaInnotech Inc.).

RESULTS

RhoA Activity Decreases as Chondrogenesis Progresses—We analyzed RhoA activity during chondrogenic differentiation of ATDC5 cells. Substantial RhoA activity was detected at day 3 of differentiation (Fig. 1), when expression of chondrogenic marker genes is relatively low (14). After 6 days in culture, in parallel to increased expression of collagen II and aggrecan (24), a significant reduction of RhoA activity is demonstrated that is maintained by day 9 of culture (Fig. 1).

The abbreviations used are: RT, reverse transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CMV, cytomegalovirus.
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**RhoA Overexpression Reduces Expression of Multiple Markers of Cartilage Development**—Previously, we have shown that stimulation of the RhoA/ROCK pathway in ATDC5 cells reduces transcript levels of Sox9, which can be rescued by inhibition of ROCK with the pharmacological compound Y27632 (14). We demonstrate here that the effects of RhoA overexpression on the Sox9 target genes collagen II and aggrecan and the transcription factors L-Sox5 and Sox6 are all decreased with RhoA overexpression. Vector-transfected cells (which demonstrate identical behavior to wild-type control ATDC5 cells (24)) display an increase in expression of mRNAs for all four genes over time (Fig. 2, black bars). ROCK inhibition with Y27632 induced enhanced expression of all genes (Fig. 2, white bars), whereas RhoA overexpression caused a reduction in the levels of transcripts for all four genes (Fig. 2, dark gray bars). The addition of Y27632 partially rescued the effects of RhoA overexpression (Fig. 2, light gray bars). These data demonstrate that RhoA signaling through ROCK suppressed expression of all chondrogenic marker genes in ATDC5 cells.

**ROCK Inhibition in Primary Micromass Cultures Results in Reduced Expression of Sox9 Target Genes**—We previously also demonstrated effects of ROCK inhibition using mouse primary limb mesenchyme cultured in high density, three-dimensional micromass cultures (14). ROCK inhibition in these cells increased transcription and mRNA levels of Sox9, similar to ATDC5 cells (14). We analyzed downstream targets of Sox9 as in the ATDC5 cell line. Fig. 3A shows that ROCK inhibition by Y27632 did not increase transcripts of collagen II as expected but rather caused a significant decrease after 3 and 4 days in culture (Fig. 3A). Examination of aggrecan transcripts revealed a similar decrease of transcripts in response to Y27632 (Fig. 3B). Since Sox9 is thought to be the major transcriptional regulator of these genes, our data suggest that Sox9 activity is negatively affected upon ROCK inhibition in the micromass culture system.

A possible explanation for the discrepancy of the observed effects on the expression of Sox9 and its target genes is the differential regulation of L-Sox5 and Sox6. Indeed, our data show that L-Sox5 (Fig. 3C) and Sox6 (Fig. 3D) transcripts are significantly decreased in micromass cultures upon ROCK inhibition.

**Cytoskeletal Modifications Alters Expression of Sox9 Downstream Targets**—Inhibition and promotion of actin polymerization by cytochalasin D and jasplakinolide, respectively, have been demonstrated to increase transcript levels of Sox9 in high density cultures of mouse limb mesenchyme (14). We demonstrate that cytochalasin D treatment decreased collagen II and aggrecan transcripts (Fig. 4, A and B) similarly to Y27632 treatment in this culture system. However, jasplakinolide treatment increased both collagen II and aggrecan mRNA levels (Fig. 4, A and B). These data suggest that although cytochalasin D and jas-
plakinolide had a similar effect in increasing transcripts of Sox9, only inhibition of actin polymerization negatively affected downstream targets of Sox9. We also demonstrate that cytochalasin D similarly reduced and jasplakinolide significantly increased transcripts of L-Sox5 and Sox6 (Fig. 4, C and D). These data suggest that the decrease in L-Sox5 and Sox6 expression is likely contributing to the observed decrease of collagen II and aggrecan mRNA levels in response to Y27632 and cytochalasin D.

**Temporal Patterns of Sox9 Phosphorylation Correlate with Expression of Sox9 Target Genes in Micromass Cultures**—To assess why the downstream targets of Sox9 were not increasing in response to higher levels of Sox9 transcripts upon ROCK inhibition, we examined Sox9 activity. Analysis of Sox9 protein levels with Y27632-treated primary cells showed similar levels to control cultures (data not shown). We examined Sox9 phosphorylation using an antibody directed to Sox9 phosphorylated on serine 181, one of the consensus cAMP-dependent protein kinase phosphorylation sites, which are known to stimulate Sox9 activity (21). In control cultures, day 2 cells displayed maximal Sox9 phosphorylation. In Y27632-treated cultures, there is a delay in Sox9 phosphorylation, and maximal levels are not achieved until day 4 of culture (Fig. 5). We had also demonstrated that cytochalasin D treatment represses expression of downstream targets of Sox9 in the micromass culture system, whereas jasplakinolide increases their transcript levels. To determine whether the phosphorylation patterns of Sox9 correspond to these discrepancies, we assayed phosphorylation of serine 181 in these cultures. Cytochalasin D treatment resulted in a similar pattern of Sox9 phosphorylation as in control cultures, but levels appeared slightly reduced by day 2 of culture (Fig. 5).

However, stabilization and promotion of actin polymerization by jasplakinolide resulted in strong Sox9 phosphorylation from day 1 of culture, thus earlier than in controls (Fig. 5). These data suggest that the decrease in expression of Sox9 target genes observed upon ROCK inhibition treatment is likely due, at least in part, to delayed and/or decreased phosphorylation of Sox9 and that early Sox9 phosphorylation is critical for maximal expression of Sox9 target genes.

**Sox9 Activity Is Decreased upon ROCK Inhibition**—To examine whether the delay in Sox9 phosphorylation and the decreased levels of L-Sox5 and Sox6 causes a functional decrease in Sox9 activity, we analyzed the effects of ROCK inhibition of a Sox9-responsive promoter in a reporter gene assay. We observed that ROCK inhibition by Y27632 resulted in a slight but significant decrease of Sox9 activity in micromass cultures (Fig. 6). A stronger reduction was seen with cytochalasin D treatment; however, a significant increase of Sox9 activity was demonstrated upon promotion of actin polymerization by jasplakinolide (Fig. 6). These data parallel the effects observed with each inhibitor with regards to transcript levels of L-Sox5, Sox6, collagen II, and aggrecan.

**Primary Chondrocyte Responses to ROCK Inhibition Resemble ATDC5 Cells**—Our data had shown opposing effects of Y27632 on chondrogenic gene expression in the two mouse culture models employed so far, ATDC5 cells and primary micromass culture. To examine whether these discrepancies were due to the transformed nature of the cell line or to differences in two- versus three-dimensional culture systems, we performed additional experiments in primary chondrocytes from mouse long bones in monolayer culture. We observed that Y27632 and cytochalasin D treatments increased the expression of collagen II and aggrecan in these cultures, resembling the effects observed in ATDC5 cells. However, the magnitude of these effects was less pronounced than in the micromass culture system, suggesting that the three-dimensional culture environment plays a significant role in the regulation of chondrogenic gene expression by ROCK inhibition.
seen in ATDC5 cells (Fig. 7). Interestingly, jasplakinolide increased only collagen II transcript levels but not aggrecan expression (Fig. 7, C and D). Sox9 activity in primary chondrocytes in monolayer culture was assessed with the use of the Sox9-responsive luciferase reporter assay. Analysis of Sox9 activity revealed that ROCK inhibition significantly increased Sox9 activity in primary cells (Fig. 8). Additionally, cytochalasin D and jasplakinolide also significantly increased Sox9 activity, but again, jasplakinolide effects were relatively weak.

**DISCUSSION**

In this study, we demonstrate that RhoA/ROCK pathway regulates chondrogenic expression in a manner that is highly dependent on the
culture model employed. The cell line ATDC5 is a model of chondrogenic differentiation, and upon stimulation with insulin, markers of differentiation are up-regulated, such as collagen II (24). We have shown here that RhoA overexpression inhibits expression of collagen II, aggrecan, L-Sox5, and Sox6 in these cells. The effects of RhoA overexpression were rescued by the addition of the ROCK inhibitor, Y27632, and ROCK inhibition of control cells resulted in an increase of all markers. We therefore show that the RhoA/ROCK pathway repressed chondrogenic gene expression in general, likely through down-regulation of Sox9 levels. Similar results were obtained in primary chondrocytes in monolayer culture, suggesting that this mechanism is not a unique feature of the ATDC5 cell line.

However, chondrocytes in micromass cultures display the opposite behavior, displaying reduced expression of Sox9 target genes despite increased levels of Sox9 mRNA. Although Sox9 protein is not elevated by Y27632 as much as the corresponding mRNA, it appears to be at least as high as in control cells. Altered protein levels can therefore not explain reduced collagen II and aggrecan expression and reduced Sox9 activity in the reporter gene assay upon Y27632 treatment. Instead, we discovered two other mechanisms that are both likely to contribute to reduce Sox9 activity: delayed Sox9 phosphorylation and reduced expression of L-Sox5 and Sox6. Serine 181 phosphorylation by protein kinase A has been shown to increase Sox9 activity (21) and is delayed by Y27632. It will be of interest to determine whether downstream mechanisms are mediating this effect. Although ROCK has been documented to directly phosphorylate transcription factors (25, 26), our data suggest that ROCK itself does not phosphorylate Sox9 as the phosphorylation and/or activity of Sox9 is not reduced by Y27632 in the other culture systems examined.

In addition, L-Sox5 and Sox6 cooperate with Sox9 to stimulate transcription (19, 20), and reduced levels of these coactivators likely contribute to the lower expression of Sox9 target genes. It should be noted that Sox9 has been shown to be essential for maximal expression of L-Sox5 and Sox6 (27), adding further complexity to this scenario. It is also interesting to note that glycosaminoglycan production is not adversely affected by Y27632 in micromass cultures (14) and appears to be independent of Sox9 activity and transcription of collagen II and aggrecan.

Although the observed disparity in the expression of Sox9 and its target genes in Y27632-treated micromass cultures were unexpected, it is not without precedent. AP-2α is a negative regulator of chondrogenesis. Overexpression of this transcription factor maintains Sox9 expres-
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sion but decreases levels of collagen II, aggrecan, L-Sox5, and Sox6 genes (28). Similarly, decreased Sox9 expression does not always correlate with decreased collagen II levels in osteoarthritic cartilage (29).

The most striking observation in the study is the difference of effects in the different models employed: ATDC5 cells and primary chondrocytes in monolayer culture on one hand and primary monolayer and primary threedimensional cultures on the other hand. In all systems, ROCK inhibition causes induction of Sox9 expression, but the effects on downstream genes are vastly different. Similar effects are observed with cytochalasin-D, an inhibitor of actin polymerization. In contrast, jasplakinolide (which stabilized existing actin filaments and thus block actin dynamics) enhances expression of chondrocyte marker genes examined in all three culture models. Thus, ROCK inhibition and blockade of actin polymerization exhibit context-specific regulation of chondrogenic gene expression, whereas stabilization of the actin cytoskeleton promotes numerous aspects of chondrogenesis independently of the culture model.

We suggest that two major differences in the culture system can account for the observed discrepancies: two-versus three-dimensional cultures of Rhō/ROCK signaling and thus differential effects of the inhibitors. Alternatively, additional pathways that collaborate with Rhō/ROCK in the control of Sox9 activity might display differential activation in two-versus three-dimensional cultures.

In addition, primary chondrocytes and ATDC5 cells are relatively pure populations of chondrogenic and prechondrogenic cells, respectively. In contrast, the embryonic limb bud cells used for micromass cultures represent a mixed population with cells able to differentiate to affect these cells as well. It has been shown that RhōA/ROCK inhibitors are controlled, in part, by receptor for cell-matrix and cell-cell interactions such as integrins (30, 31) and cadherins (32, 33). It is feasible that differences in these interactions between monolayer and three-dimensional cultures result in differential patterns of RhōA/ROCK signaling and thus differential effects of the inhibitors. Alternatively, additional pathways that collaborate with RhōA/ROCK in the control of Sox9 activity might display differential activation in two-versus three-dimensional cultures.

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REFERENCES

1. Karsenty, G. (1999) Genes Dev. 13, 3037–3051
2. Shum, L., and Nuckolls, G. (2002) Arthritis Res. 4, 94–106
3. Horton, W. A. (1993) In Extracellular Matrix and Heritable Disorder of Connective Tissue (Royce, P. M., and Steinman, B., eds), pp. 73–84, Alan R. Liss, New York
4. Bi, W., Deng, J. M., Zhang, Z., Behringer, R. R., and de Crombrugghe, B. (1999) Nat. Genet. 22, 85–99
5. Li, S. W., Procop, D. J., Helminen, H., Fassler, R., Lapvetelainen, T., Kiraly, K., Peltrari, A., Arakoski, J., Lui, H., and Aita, M. (1995) Genes Dev. 9, 2821–2830
6. Doege, K. J. (1999) In Guidebook to the Extracellular Matrix, Anchor and Adhesion Proteins, Second Ed. (Kreis, T., and Vale, R., eds) pp. 359–361, A Sambrook and Tooze Publication at Oxford University Press, Oxford
7. Brown, P. D., and Benya, P. D. (1988) J. Cell Biol. 106, 171–179
8. Amano, M., Chihara, K., Kimura, K., Fukuta, Y., and Nakamura, N. (1997) Science 275, 1304–1311
9. Zanetti, N. C., and Solursh, M. (1984) J. Cell Biol. 99, 115–123
10. Takai, Y., Sasaki, T., Tanaka, K., and Nakashima, H. (1995) Trends Biochem. Sci. 20, 227–231
11. Mamamoto, A., Huang, S., Moore, K., Oh, P., and Inghber, D. E. (2004) J. Biol. Chem. 279, 26323–26330
12. Nober, C. D., Hawking, P., Stephens, L., and Hall, A. (1995) J. Cell Sci. 108, 225–233
13. Kobayashi, K., Takahashi, M., Matsushita, N., Miyazaki, J.-i., Koike, M., Yaginuma, H., Osumi, N., Kaibuchi, K., and Kobayashi, K. (2004) J. Neurosci. 24, 3480–3488
14. Woods, A., Wang, G., and Beier, F. (2005) J. Biol. Chem. 280, 11626–11634
15. Healy, C., Uwahnoho, D., and Sharpe, P. T. (1999) Dev. Dyn. 215, 69–78
16. Bell, D. M., Leung, K. K. H., Wheatley, S. C., Ng, L. J., Zhou, S., Wing Ling, K., Har Sham, M., Koopman, P., Tam, P. P. L., and Cheah, K. S. H. (1997) Nat. Genet. 16, 174–179
17. Sekiya, I., Tsuji, K., Koopman, P., Wang, A. C., Yamada, Y., Shimomori, K., Nifuji, A., and Noda, M. (2000) J. Biol. Chem. 275, 10738–10744
18. Smits, P., Li, P., Mandel, J., Zhang, Z., Deng, J. M., Behringer, R. R., de Crombrugghe, B., and Lefebvre, V. (2000) Cell 101, 277–290
19. Lefebvre, V., Li, P., and de Crombrugghe, B. (1998) EMBO J. 17, 5718–5733
20. Shum, L., and Nuckolls, G. (2002) J. Biol. Chem. 277, 549–555
21. Huang, W., Zhou, X., Lefebvre, V., and de Crombrugghe, B. (2000) Mol. Cell Biol. 20, 4419–4418
22. Weston, A. D., Chandraratna, R. A. S., Torchia, J., and Underhill, T. M. (2002) J. Cell Biol. 158, 39–51
23. Stanton, L. A., Sabari, S., Sampaio, A. V., Underhill, T. M., and Beier, F. (2004) Biochem. J. 378, 53–62
24. Wang, G., Woods, A., Sabari, S., Pagnotta, L., Stanton, L. A., and Beier, F. (2004) J. Biol. Chem. 279, 13205–13214
25. Anwar, K. N., Fazal, F., Malik, A. B., and Rahman, A. (2004) J. Immunol. 173, 6965–6972
26. Kamaraju, A. K., and Roberts, A. B. (2005) J. Biol. Chem. 280, 1024–1036
27. Akiyama, H., Chabosossier, M. C., Martin, J. F., Schell, A., and de Crombrugghe, B. (2002) Genes Dev. 16, 2683–2693
28. Huang, Z., Xu, H., and Sandell, L. (2004) J. Bone Miner. Res. 19, 245–255
29. Aigner, T., Gebhard, P. M., Schmid, E., Bau, B., Harley, V., and Posch, E. (2003) Matrix Biol. 22, 363–372
30. Clancy, R. M., Rediske, J., Tang, X., Niijii, N., Frenkel, S., Phillips, M., and Abramson, S. B. (1997) J. Clin. Invest. 100, 1789–1796
31. Takahashi, I., Onodera, K., Sasano, Y., Miyazaki, J., Bae, J. W., Mitani, H., Kagayama, M., and Mitani, H. (2003) Eur. J. Cell Biol. 82, 182–192
32. Oberlander, S. A., and Tuan, R. S. (1994) Development (Can.ah) 120, 177–187
33. Delise, A. M., and Tuan, R. S. (2002) Dev. Dyn. 225, 195–204
34. Weston, A. D., Sampaio, A. V., Ridgeway, A. G., and Underhill, T. M. (2003) J. Cell Sci. 116, 2885–2893
35. Seifert, R. (1994) Cell Tissue Res. 277, 599–555
36. Shakibaee, M., and De Souza, F. P. (1997) Cell Biol. Int. 21, 75–86
37. Nishiyama, T., Kii, I., and Kudo, A. (2004) J. Biol. Chem. 279, 47311–47319
38. Zhao, Z., and Rivkees, S. A. (2004) Dev. Biol. 275, 183–191
39. McBeth, R., Pirone, D. M., Nelson, C. M., Bhadriraju, K., and Chen, C. S. (2004) Dev. Biol. 26, 483–495
40. Nuttall, M. E., and Gimble, J. M. (2004) Curr. Opin. Pharmacol. 4, 290–294
41. Di Nino, D. L., Long, F., and Linsemayr, T. F. (2001) Dev. Biol. 240, 433–442
42. Colnot, C., Lu, C., Hu, D., and Helms, J. A. (2004) Dev. Biol. 269, 55–69

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