Abstract

**Background:** Wnt signaling is implicated in many developmental decisions, including stem cell control, as well as in cancer. There are relatively few target genes known of the Wnt pathway.

**Results:** We have identified target genes of Wnt signaling using microarray technology and human embryonic carcinoma cells stimulated with active Wnt protein. The ~50 genes upregulated early after Wnt addition include the previously known Wnt targets Cyclin D1, MYC, ID2 and βTRCP. The newly identified targets, which include MSX1, MSX2, Nucleophosmin, Follistatin, TLE/Groucho, Ubc4/5E2, CBP/P300, Frizzled and REST/NRSF, have important implications for understanding the roles of Wnts in development and cancer. The protein synthesis inhibitor cycloheximide blocks induction by Wnt, consistent with a requirement for newly synthesized β-catenin protein prior to target gene activation. The promoters of nearly all the target genes we identified have putative TCF binding sites, and we show that the TCF binding site is required for induction of Follistatin. Several of the target genes have a cooperative response to a combination of Wnt and BMP.

**Conclusions:** Wnt signaling activates genes that promote stem cell fate and inhibit cellular differentiation and regulates a remarkable number of genes involved in its own signaling system.

Background

There is currently great interest in using extracellular signaling proteins to influence the gene expression program and differentiation of embryonic cells, in particular embryonic stem cells. Of the factors that contribute to specifying cell fate during development, Wnt proteins are among the most attractive candidates to use in such *in vitro* experiments. Wnt proteins control numerous aspects of development, ranging from stem cell control to differentiation and cell polarity [1,2]. It has been problematic however to test directly whether Wnt proteins can be used as reagents in cell culture, because working with soluble Wnt proteins is difficult and few cell lines are known to respond to Wnt proteins. Wnt signal transduction proceeds through a complex series of protein interactions, initiated by binding of the Wnt protein to cell surface receptors.
The regulation of the GSK3 protein kinase and its substrate (Frizzled and LRP [3–5]) which generates a signal to downstream components. A key event in signaling is the activation of Wnt (data not shown; Figure 2A). To stimulate the NCCIT cells with Wnt, we used tissue culture medium containing active Wnt-3A protein produced by mouse L cells (Wnt-3A CM [8]), in comparison to control conditioned medium (CCM). In initial experiments, we found that Wnt-3A protein elevates the levels of β-catenin 5–10 fold (Figure 4); and that a transiently transfected TCF reporter construct is activated 3–4 fold (not shown).

To identify target genes, we applied Wnt-3A CM or CCM to the NCCIT cells and isolated RNA at several time points. We performed differential hybridization to microarray slides containing approximately 23,000 spots of human cDNAs, using RNA from CCM-exposed cells as a reference. Figure 1 shows a cluster analysis of the hybridization results. We found approximately 50 genes that were upregulated between 2 and 10 fold by Wnt-3A CM whereas a few genes were repressed, i.e. expressed at lower levels in the Wnt-3A-treated cells. The latter group consisted mostly of ESTs of unknown genes and has yet to be characterized.

We verified Wnt-induced expression of several genes with Northern blots (Figure 2). In all cases tested, we confirmed the increase observed in the microarray; in some cases we found even higher differences in expression. For example, MSX2, ID2, REST/NRSF, FZD7 and Follistatin were confirmed by Northern analysis to be significantly elevated by Wnt-3A CM after two hours (Figure 2A).

None of these genes was induced by the CCM, suggesting that they are Wnt-specific. It remained possible however that the observed changes in gene expression were influenced by other differences between Wnt-3A CM and CCM. For example, Wnt-3A could have induced the expression of another secreted factor in the mouse L cells which would then be present only in the Wnt-3A CM. We addressed this possibility by specifically removing active Wnt-3A from the conditioned medium, using a combination of the ligand binding domain of the Drosophila Wnt/Wingless receptor Frizzled-2 fused to Alkaline Phosphatase [3,9] and Wnt-3A specific antibodies. This process...
dure specifically depleted active Wnt-3A from the medium. As expected, neither β-catenin levels nor a TCF reporter gene were elevated by the depleted CM (not shown). The depleted medium also failed to induce MSX1, MSX2 and ID2 and CyclinD1 expression as assayed by Northern blots (Figure 2B shows loss of MSX1) and in a DNA microarray analysis with CCM as a reference (not shown). Similarly, adding Dickkopf protein, an inhibitor of Wnt signaling that binds to the co-receptor LRP [10], to the medium abrogated the transcriptional response (data not shown). We conclude that the transcriptional response to Wnt-3A CM is indeed controlled by the Wnt-3A protein rather than by other factors.

**Cooperativity between Wnt-3A and BMP**

Several of the Wnt target genes had been identified previously as targets of BMP signaling [11]. We were therefore interested in comparing the effects of BMP and Wnt-3A and examining their combined effects. As has been reported before for other cells [11], MSX1, MSX2 and ID2 are elevated by BMP-4 in NCCIT cells (Figure 3). When Wnt-3A and BMP-4 were combined, gene expression was increased to yet higher levels. MSX1 expression was markedly elevated by the combination of BMP-4 and Wnt-3A. Similar but less dramatic effects were found for ID2 and MSX2 (Figure 3). Hence, BMP-4 and Wnt-3A appear to have an additive or perhaps synergistic effect on target gene expression. However, when the time courses of Wnt- and BMP-induced gene expression were compared, it appeared that BMP-4 acts as soon as at 30 minutes, but that Wnt takes 2 hours to have an effect (Figure 3). Hence, Wnt-induced changes in gene expression are slower than BMP, even on the same target genes and in the same cell type.

**Wnt signaling is blocked by cycloheximide**

The delay in transcriptional response to soluble Wnt raised the concern that the genes we identified might not be primary targets of Wnt but dependent on the prior induction of other genes. Such concerns are usually addressed by blocking protein synthesis in cells because the expression of primary target genes, or so-called immediate early genes, should not be sensitive whereas secondary targets should be blocked. This approach is not readily applicable to the Wnt response because the critical step in signal transduction is the block in β-catenin phosphorylation and degradation, leading to the accumulation of newly-synthesized β-catenin. Whether Wnt target gene activation is cycloheximide sensitive has never been tested however. When we incubated cells with cycloheximide, the accumulation of β-catenin by Wnt-3A was blocked; and Wnt targets, like MSX2, were not elevated (Figure 4). The inhibitory effect was also seen for MSX1, ID2, Follistatin, Versican and Cyclin D1 (not shown). In contrast, cycloheximide did not block induction of target genes by BMP (Figure 4), the mechanism of which does not involve protein accumulation [12]. These findings underscore the importance of β-catenin protein elevation in the response to Wnt. This unique aspect of Wnt signaling may explain its relatively slow effects on target genes – protein synthesis is slower than protein phosphorylation or other signal transduction events.
The requirement for protein synthesis in Wnt signaling means that we cannot use cycloheximide sensitivity as a criterion to discriminate between primary and secondary targets.

**The Follistatin promoter is dependent on a TCF binding site**

In the Wnt target gene promoters that are functionally mapped (MSX1, MSX2, ID2, ID3, Follistatin, REST/NRSF, Versican) we invariably found TCF binding sites. Moreover, we found that a luciferase reporter gene, placed under the control of the Follistatin promoter, was activated by Wnt-3A protein added to transiently transfected NCCIT cells (Figure 5). Mutating the single TCF binding site on the Follistatin promoter eliminated the response. These data suggest that Follistatin is activated directly through the Wnt signal transduction pathway and not by another pathway. Because the kinetics of Follistatin activation by Wnt are similar to that of the other target genes (Figure 2A), we suggest that there are no "earlier" targets, i.e. all identified targets are direct.

**Discussion**

The overall pattern of gene expression observed in response to Wnt has important implications for the role of Wnt signaling in development and cancer. Of particular interest are the ID, MSX, and REST/NRSF genes. Expression of each of these genes can interfere with differentiation of cells. ID2 was recently shown also to be a target of activated β-catenin in colon carcinoma cells [13]. It is a
Several of the Wnt targets genes encode components of the Wnt signaling transduction system. The increased expression of βTrCP/slimb [26] and the ubiquitin conjugating enzyme Ubc4/5E2 (Figure 1), both involved in degradation of β-catenin [27], could act as a negative feedback loop in Wnt signaling. Moreover, Frizzled receptors, Versican and other proteoglycans which contribute to Wnt protein activity are also controlled by Wnt signaling, as previously described in Drosophila [28,29]. At the level of transcriptional regulation, we found up-regulation of CBP/P300, which partners with β-catenin [30,31] but also TLE/Groucho, which is an inhibitor of TCF function [32]. These responses suggest a rich system of homeostasis and feedback control mechanisms, but they complicate the interpretation of experimental studies of Wnt signal transduction in which the time course of induction cannot be controlled easily.

The delayed response to Wnt signaling raises interesting developmental issues. Timing of gene expression is very important during embryogenesis. A slow response is likely to be an important feature of the activity of Wnts as morphogens, when not only the concentration but also the duration of the stimulus is important. In considering these issues, it is of interest to note that in the early C. elegans embryo, Wnt signaling proceeds through a non-canonical pathway, through phosphorylation of the TCF homolog but not using the increase in β-catenin [33]. Cell divisions and polarization in the worm embryo occur on a time scale of minutes, and the different mechanism of Wnt signaling in that context may be more suitable than the inherently slower translation-dependent β-catenin increase.

Conclusions
We found that Wnt signaling can activate genes that promote stem cell fate and inhibit cellular differentiation; and regulates a remarkable number of genes involved in its own signaling system. The overall pattern of gene expression provides clues to the diverse functions of Wnt proteins in cancer, cell fate decisions and other developmental events.

Methods
Cells
Mouse L cells secreting Wnt-3A were established in our lab, by transfecting expression constructs in which the Wnt-3A cDNA [34] is driven by the PGK promoter. In order to get cells secreting relatively high amounts of Wnt-3A, we selected clones from the transfected cell population. The tissue culture supernatant was added to NCCIT cells [7].

The MSX and ID genes are also activated by BMP [11] and we found indeed synergistic effects of Wnt plus BMP. There are several examples of interactions between BMP and Wnt family members in vivo, particularly in Drosophila [21] and this cross-talk adds to the potential of these pathways for cell type specification. However, Wnt signaling also promotes the expression of such genes as Cyclin D1 [22] and MYC [23], which are not elevated by BMP. The ability to block differentiation and simultaneously promote cell division is a distinctive characteristic of Wnt signaling which could be instrumental in the role of Wnts as stem cell growth factors, such as in the crypt of the colon, and in carcinogenesis [2,24,25].

Figure 4
A) Western blot demonstrating cycloheximide effects of Wnt-3A mediated accumulation of β-catenin. NCCIT cells were stimulated for 4 hours with CCM (C) or Wnt-3A CM (Wnt) in the presence (20 microgram/ml) or absence (0) of cycloheximide. Note that β-catenin does not accumulate in response to Wnt-3A CM when cycloheximide is present. B) Northern analysis demonstrating that MSX2 induction by Wnt-3A is abolished in the presence of cycloheximide. Cells were stimulated for 4 hours with CCM (C), BMP-4 or Wnt-3A CM (Wnt) in the presence (20 microgram/ml media) or absence of cycloheximide. As expected, BMP-4 mediated induction of MSX2 is not affected by cycloheximide which contrasts with the inhibition of Wnt-3A mediated induction of MSX2 in the presence of cycloheximide. GAPDH was used to verify equal loading. The inhibitory effect was also seen for MSX1, ID2, Follistatin, Versican and Cyclin D1 (not shown).

general inhibitor of the bHLH proteins such as MyoD and E12/E47 [14] and can interact with the tumor suppressor RB1 [15]. MSX1 expression can revert differentiated myotubes into an undifferentiated state [16]. REST/NRSF is known to block neural differentiation by binding to and inhibiting the expression of neuronal genes [17,18]. To what extent these genes are controlled by Wnt signaling in intact animals or in other cell types is not clear yet, but we do note that MSX mutants have phenotypes similar to LEF/TCF mutants [19,20].

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Response of a reporter containing the Follistatin promoter to Wnt-3A protein in NCCIT cells [37]. The Follistatin promoter [36] contains one putative TCF binding site (CTTGTGAT). This promoter was linked to luciferase and transfected in NCCIT cells, which were then exposed to Wnt-3A CM and CCM for 8 hours. This assay showed a significant increase in activity when cells were stimulated directly with Wnt-3A CM or co-transfected with activated β-catenin (not shown). This effect was abrogated by co-transfection of dominant-negative TCF-4 or axin (not shown). When the TCF site within the Follistatin promoter was mutated into CATCGAT, the Wnt-3A response was abolished.

Depletion of Wnt protein
The medium was specifically depleted of Wnt-3A by adding an excess of a fusion protein containing the cysteine-rich domain of the Frizzled2 receptor of Drosophila (which binds to Wnt-3A protein) and alkaline phosphatase. [9]. By adding antibody to alkaline phosphatase, all of the detectable Wnt-3A protein could be removed from the medium. In addition, an antibody to Wnt-3A itself was used for depletion, although it worked less efficiently than the Frizzled fusion protein

Mutating the Follistatin promoter
The Follistatin promoter [36] contains one putative TCF binding site (CTTGTGAT) at position -223 to -217 from the start of transcription. This site was mutated into CATCGAT, which led to abrogation of the Wnt-3A response.

Authors' contributions
JW, ME and JP performed the experiments. JW and ME made equal contributions to this work. PB and RN supervised the work. All authors read and approved the final manuscript.

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References
1. Cadigan K, Nusse R: Wnt signaling: a common theme in animal development. Genes & Dev. 1997, 11:3286-3305
2. Taipale J, Beachy PA: The Hedgehog and Wnt signalling pathways in cancer. Nature 2001, 411:349-54
3. Bhanoit P, Brink M, Harryman Samos C, Hsieh JC, Wang YS, Macke JP, Andrew D, Nathans J, Nusse R: A new member of the frizzled family from Drosophila functions as a Wingless receptor. Nature 1996, 382:225-230
4. Wehrli M, Dougan ST, Caldwell K, O’Keefe L, Schwartz S, Vaizel-Ohayon O, Schejter E, Tomlinson A, DiNardo S: arrow encodes an LDL-receptor-related protein essential for Wingless signaling. Nature 2000, 407:527-30
5. Moon RT, Brown JD, Yang-Snyder JA, Miller JR: Structurally related receptors and antagonists compete for secreted Wnt ligands. Cell 1997, 88:725-8
6. Polakis P: More than one way to skin a catenin. Cell 2001, 105:563-6
7. Damjanov I, Horvat B, Gisaz Z: Retinoic acid-induced differentiation of the developmentally pluripotent human germ cell tumor-derived cell line, NCCIT. Lab Invest 1993, 68:220-32
8. Willert K, Shibamoto S, Nusse R: Wnt-induced dephosphorylation of Axin releases b-catenin from the Axin complex. Genes Dev 1999, 13:1768-1773
9. Rulifson EJ, Wu CH, Nusse R: Pathway specificity by the bifunctional receptor frizzled2 is determined by affinity for wingless. Mol Cell 2001, 61:17-26
10. Mao B, Wu W, Li Y, Hoppie D, Stannek P, Glinka A, Niehrs C: LDL-receptor-related protein is a receptor for Dickkopf proteins. Nature 2001, 411:321-5
11. Hollnagel A, Oehlmann V, Heymer J, Ruther U, Nordheim A: Id genes are direct targets of bone morphogenetic protein induction in embryonic stem cells. J Biol Chem 1999, 274:19838-45
12. Massague J, Wotton D: Transcriptional control by the TGF-beta/Smad signaling system. Endo J 2000, 19:1745-54
13. Rockman SP, Currie SA, Ciavarella M, Vincan E, Dow C, Thomas RJ, Phillips WA: Id2 is a target of the beta-catenin/T cell factor pathway in colon carcinoma. J Biol Chem 2001, 276:45113-9
14. Noron JD, Deed RW, Crags G, Sabitizky F: Id helix-loop-helix proteins in cell growth and differentiation. Trends Cell Biol 1998, 8:58-65
15. Lasorella A, Noseda M, Beyna M, laronone A: Id2 is a retinoblastoma protein target and mediates signalling by Myc oncoproteins. Nature 2000, 407:592-8
16. Odelberg SJ, Kolhoff A, Keating MT: Dedifferentiation of Mammalian Myotubes Induced by MDI. Cell 2000, 103:1099-1109
17. Chong JA, Tapia-Ramirez J, Kim S, Toledo-Aral JI, Zheng Y, Boutros MC, Altshuller YM, Frohman MA, Krainer SD, Mandel G: REST: a mammalian silencer protein that restricts sodium channel gene expression to neurons. Cell 1995, 80:949-57
18. Schoenherr CJ, Anderson DJ: The neuron-restrictive silencer factor (NRSF) is a coordinate repressor of multiple neuron-specific genes. Science 1995, 267:1360-3
19. van Genderen C, Okamura RM, Farinas I, Quo RG, Parslow TG, Bruhn L, Grosschedl R: Development of several organs that require inductive epithelial-mesenchymal interactions is impaired in LEF-1-deficient mice. Genes Dev 1994, 8:269-1-2703

20. Satokata I, Ma L, Ohshima H, Bei M, Woo I, Nishizawa K, Maeda T, Takano Y, Uchiyama M, Heaney S, et al: Msx2 deficiency in mice causes pleiotropic defects in bone growth and ectodermal organ formation. Nat Genet 2000, 24:391-5

21. Thiringer F, Cohen SM, Bienz M: Dissection of an indirect autoregulatory response of a homeotic drosophila gene. EMBO J 1993, 12:2419-2430

22. Tetsu O, McCormick F: Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. Nature 1999, 398:422-6

23. He TC, Sparks AB, Rago C, Hermeking H, Zawel L, da Costa LT, Morin PJ, Vogelstein B, Kinzler KW: Identification of c-MYC as a target of the APC pathway. Science 1998, 281:1509-12

24. Korinek V, Barker N, Moerer P, van Donselaar E, Huls G, Peters PJ, Clevers H: Depletion of epithelial stem-cell compartments in the small intestine of mice lacking Tcf-4. Nat Genet 1998, 19:379-83

25. Bienz M, Clevers H: Linking colorectal cancer to Wnt signaling. Cell 2000, 103:311-20

26. Spiegelman VS, Slaga TJ, Pagano M, Ronai Z, Fuchs SY: Wnt/beta-catenin signaling induces the expression and activity of betaTrCP ubiquitin ligase receptor [In Process Citation]. Mol Cell 2000, 5:877-82

27. Yaron A, Hatzubai A, Davis M, Lavon I, Amit S, Manning AM, Anderson JS, Mann M, Mercurio F, Ben-Neriah Y: Identification of the receptor component of the IkappaBa-ubiquitin ligase. Nature 1998, 396:590-4

28. Baeg GH, Lin X, Khare N, Baumgartner S, Perrimon N: Heparan sulfate proteoglycans are critical for the organization of the extracellular distribution of Wingless. Development 2001, 128:87-94

29. Sato A, Kojima T, Ui-Tei K, Miyata Y, Saigo K: Dfrizzled-3, a new Drosophila Wnt receptor, acting as an attenuator of Wingless signaling in wingless hypomorphic mutants. Development 1999, 126:441-30

30. Takemaru KI, Moon RT: The transcriptional coactivator CBP interacts with beta-catenin to activate gene expression. J Cell Biol 2000, 149:249-54

31. Hecht A, Vlemingck K, Stemmler MP, van Roy F, Kemler R: The p300/CBP acetyltransferases function as transcriptional coactivators of beta-catenin in vertebrates. Embo J 2000, 19:1839-50

32. Roose J, Molemaar M, Peterson J, Hurenkamp J, Brantjes H, Moerer P, van de Wetering M, Destree O, Clevers H: The Xenopus Wnt effector XTcf-3 interacts with Groucho-related transcriptional repressors. Nature 1998, 395:608-12

33. Rocheleau CE, Yasuda J, Shin TH, Lin R, Sawa H, Okano H, Priess JR, Davis RJ, Mello CC: WRM-1 activates the LIT-1 protein kinase to transduce anterior/posterior polarity signals in C. elegans. Cell 1999, 97:717-26

34. Roelink H, Nusse R: Expression of two members of the Wnt gene family during mouse development; restricted temporal and spatial patterns in the developing neural tube. Genes Dev 1991, 5:381-388

35. Eisen MB, Spellman PT, Brown PO, Botstein D: Cluster analysis and display of genome-wide expression patterns. Proc Natl Acad Sci U S A 1998, 95:14863-8

36. Miyangka K, Shimazaki S: Structural and functional characterization of the rat follistatin (activin-binding protein) gene promoter. Mol Cell Endocrinol 1993, 92:99-109

37. Winters SJ, Dalkin AC, Tsuji T: Evidence that pituitary adenylyl cyclase activating polypeptide suppresses follicle-stimulating hormone-beta messenger ribonucleic acid levels by stimulating follistatin gene transcription. Endocrinology 1997, 138:4324-9