Regulation of Differentiation of HC11 Mouse Breast Epithelial Cells by the Signal Transducer and Activator of Transcription-3

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Abstract. *Background/Aim:* The differentiation of the mouse breast epithelial cell line HC11 is known to require confluence as well as the addition of hydrocortisone, insulin and prolactin. *Materials and Methods:* Since confluence, which triggers the engagement of the cell-to-cell adhesion molecule E-cadherin, induces a dramatic increase in the activity of signal transducer and activator of transcription-3 (Stat3), we examined the role of Stat3 in HC11 cell differentiation. *Results:* Stat3 inhibition abolished differentiation, indicating that Stat3 activity is critically required. However, expression of the mutationally activated form of Stat3 (Stat3C), rather than promoting, it was found to block cell differentiation, even when expressed in low levels, and in the absence of full neoplastic conversion. *Conclusion:* The strength of the E-cadherin/Stat3 signal is key for the outcome of the differentiation process.

The mammary gland provides a useful model to study epithelial cell differentiation. The HC11 line, which was derived from mouse breast at mid-gestation, has been used extensively in differentiation studies because it behaves like the normal mammary epithelium (1), in that it can differentiate and produce milk proteins in response to lactogenic stimulation at confluence (2).

The signal transducer and activator of transcription-3 (Stat3) is activated by receptor and non-receptor tyrosine kinases, and

cytokines, such as members of the IL6 family (3). Stat3 activation involves its binding to phosphotyrosine (tyr) sites of activated receptors through its SH2 domain. Stat3 is subsequently phosphorylated at a critical phosphotyrosine (tyr-705) by the receptor itself or associated Jak or Src kinases, dimerizes through reciprocal SH2-tyr interaction and migrates to the nucleus, where it binds to target sequences on the DNA (4). This leads to the transcriptional activation of specific genes promoting survival, such as *Mcl-1*, *Bcl-xL* or *survivin*, or proliferation, such as *myc* and *cyclin D* and others (4). Stat3 is found to be overactivated in a number of cancers (5, 6), and is required for transformation by a number of oncogenes, such as Src (7) and the Simian virus 40 large tumor antigen (8), while in an activated form (Stat3C), Stat3 is able to transform cultured cells and induce tumors in nude mice. The latter points to an etiologic role for Stat3 in cancer (9).

In addition to this mechanism, results from our lab and others have demonstrated that engagement of cadherins, as induced by confluence of cultured cells, triggers a striking increase in Stat3 activity (10, 11), through the Rac and Cdc42, small GTPases (10, 12). In fact, it has been shown that cadherin engagement *per se* can directly increase Rac and Stat3 activity, even in the absence of cell to cell contact, as shown by the fact that growing cells on surfaces coated with E-cadherin (10) or cadherin-11 (11) causes a dramatic increase in Stat3 activity.

Since confluence-induced growth arrest is an established prerequisite for HC11 cell differentiation, we recently examined the role of Rac. The results demonstrated that cRac1 is critically required for HC11 differentiation, and that expression of low levels of a mutationally activated form of cRac1 (Rac^{V12}) actually increased differentiation (13). Since Stat3 is an effector of Rac, in the present communication we examined the role of Stat3 upon HC11 differentiation. Interestingly, our results indicate that, despite the fact that Stat3 was also required for the differentiation of HC11 cells, in a manner similar to cRac1, mutationally activated Stat3C

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was found to block differentiation, even when expressed at low levels. Therefore, although Stat3 operates in the same pathway emanating from E-cadherin and is a downstream target of Rac, in an activated form induced by mutation, unlike Rac^{V12}, Stat3C does not promote differentiation.

Materials and Methods

Cell culture and differentiation induction. HC11 cells differentiate and produce β -casein, a major milk protein, when they are treated with hydrocortisone, insulin and prolactin (HIP medium) at confluence in a medium lacking EGF, a differentiation inhibitor. HC11 cells were grown in RPMI-1640 medium with 10% fetal bovine serum, 5 μ g/ml insulin (Sigma, MO, USA, Cat. #91077C) and 10 ng/ml epidermal growth factor (EGF; Sigma #E9644). For differentiation induction, at 100% confluence, cells were grown for 24 h in the same medium but lacking EGF (day 0), followed by growth in HIP medium [1 μ g/ml hydrocortisone (Sigma, Cat. #H0888), 5 μ g/ml insulin and 5 μ g/ml prolactin (Sigma Cat. #L6520) in RPMI-1640 with 10% fetal calf serum, without added EGF] for up to 10 days, as described in (13). Cells grown to the same densities and EGF-starved but not treated with HIP served as negative controls. To make sure that the cells were not starved from nutrients, HIP medium was changed every two days.

For Stat3 inhibition, cells were treated with low levels (1 μ M) of the CPA7 Stat3 inhibitor [inhibition 15 $\pm$ 5% (13-15)], or 5 μ M SH4-54 [inhibition ~20% (15-17)], or 100 μ M S3I-201 [inhibition ~20% (18, 19)]. All inhibitors were added at the time of EGF starvation (day 0) for 24 h, followed by HIP medium addition in the absence of inhibitor, and cells were allowed to differentiate for 10 days thereafter as above.

Retroviral vectors. The retroviral MSCV-Stat3C vector, which contains an internal ribosome entry site (IRES) linked to green fluorescence protein (GFP), was a gift from Dr. Daniel Link (20). The EF.Stat3C.Ubc.GFP lentiviral vector was a gift from Dr. Linzhao Chang (Addgene Cat. #24983 MA, USA) (21).

Expression was measured by Western blotting for total Stat3 and by transcriptional activity, using HC11 cells expressing high Stat3 levels using the MSCV-Stat3C vector [line HC11-Stat3C (10)] as a positive control.

Cell lysis and western blotting. Cells were scraped from the petri dishes into an 1.8 ml Eppendorf tube, washed with cold PBS by centrifugation and lysed with 1% NP-40 buffer containing protease and phosphatase inhibitors as previously described (22). Gels were probed for Stat3-ptyr705 (Cell Signalling Technology, MA, USA, rabbit monoclonal antibody D3A7, Cat. #9145), total Stat3 (Cell Signalling Technology, rabbit monoclonal antibody 79D7, Cat. #4904), β -casein (goat, Santa Cruz Biotech, TX, USA Cat. #sc-17971), E-cadherin (mouse, Invitrogen, CA, USA, Cat. #33-400), or β -actin (mouse monoclonal, Cell-signaling technology Cat. #3700) as a loading control. Luciferase assays were performed on total cell extracts as previously described (10, 23).

Anchorage-independence assays. They were performed as described (13), with HC11 cells expressing high Stat3C levels [line HC11-Stat3C (10)] as a positive control. Trypsinised cells were suspended in 0.3% low melting point agarose for 20 days at 37°C/5% CO₂. At

Figure 1. Differentiation of HC11 cells increases Stat3-ptyr705 levels. After reaching confluence in complete medium, HC11 cells were grown in the absence of EGF for 24 h, then they were induced to differentiate with HIP addition for the indicated number of days (lanes 5-8), or not (lanes 1-4). Detergent cell extracts were probed for Stat3-ptyr705, β -casein or β -actin as a loading control, as indicated. NE: Control cultures, grown in the absence of EGF, but not induced to differentiate. Numbers at the right refer to molecular weight markers.

that time, cells were photographed under phase-contrast illumination using an Olympus IX70 microscope (Olympus Corporation, Tokyo, Japan). For quantitation, colonies were stained with 0.05% crystal violet (Sigma Cat. #C3886) dissolved in 20% methanol and were counted on photographs of cells at 140 $\times$ magnification. Anchorage-independent growth was expressed as a percentage of total cell count. All experiments were performed at least three times with 3, 3 cm petris per line, counting at least 200 cells from each petri in different fields.

Results

Differentiation increases Stat3-ptyr705 levels. We and others have previously demonstrated that cadherin engagement, as occurs in confluent cultured cells, triggers a dramatic increase in Stat3-ptyr705 expression and activity in a variety of cellular systems, including HC11 [(10) reviewed in (24, 25)]. Since cell density is a prerequisite for the differentiation of HC11 cells, we now examined the effect of differentiation *per se* upon Stat3-ptyr705 levels. HC11 cells were grown and induced to differentiate, as described in Materials and Methods.

As previously demonstrated (10) cell density from 50% of confluence to 2 days post 100% confluence causes a dramatic increase in Stat3-ptyr705. However, as shown in Figure 1, HIP addition to cells grown to 100% confluence caused an approximately 2-fold increase in Stat3-ptyr705 levels, compared to EGF-starved, non-HIP-treated cells, indicating that HIP addition, and possibly differentiation, causes an increase in Stat3-ptyr705. As expected, probing for

Figure 2. (A) Stat3 inhibition with 1 μ M CPA7 on day 0 prior to induction of differentiation completely blocks differentiation of HC11 cells. HC11 cells were grown in the absence of EGF for 24 hours, then induced to differentiate with HIP medium addition for the indicated number of days (lanes 6-9 and 15-19), or not (lanes 1-5 and 10-14). Cells were treated with 1 μ M CPA7, Stat3 inhibitor (lanes 10-19) for 24 h prior to HIP addition, or the DMSO carrier (lanes 1-9). Detergent cell extracts were probed for β -casein or β -actin as a loading control, as indicated. NE: Control cultures, grown in the absence of EGF, but not induced to differentiate. Note the dramatic reduction in β -casein levels upon CPA7 treatment. Lanes 20-22: Stat3 inhibition with 5 μ M SH4-54 or 100 μ M S3I-201 on day 0 prior to induction of differentiation blocks differentiation of HC11 cells. Cells were grown in the absence of EGF then induced to differentiate as above. Cells were treated with 5 μ M SH4-54 (lane 21) or 100 μ M S3I-201 (lane 22) for 24hrs prior to HIP addition and 10 days later lysates were probed for β -casein or β -actin, as indicated. Note the reduction in β -casein levels upon treatment with the inhibitors (lanes 21, 22), compared to untreated control (DMSO carrier, lane 20). Numbers at the right refer to molecular weight markers. (C) Stat3 inhibition with 1 μ M CPA7 on day 5 following differentiation induction only partially reduces differentiation of HC11 cells. HC11 cells were grown in the absence of EGF for 24 hours, then they were induced to differentiate with HIP addition for the indicated number of days (lanes 5-8 and 14-17), or not (lanes 1-4 and 9-13). Cells were treated with 1 μ M CPA7, Stat3 inhibitor (lanes 9-17) for 24 h on day 5 following HIP addition, or not (lanes 1-8). Detergent cell extracts were probed for β -casein or β -actin as a loading control, as indicated. NE: Control cultures, grown in the absence of EGF, but not induced to differentiate. Numbers at the right refer to molecular weight markers. Note the relatively small reduction in β -casein upon CPA7 treatment on day 5 compared to day 0 in Figure 2A, above.

casein showed high levels of this protein upon HIP treatment, while it was undetectable in untreated controls (lanes 3-4 versus 7-8).

Stat3 is required for HC11 cell differentiation. Since Stat3-ptyr705 levels are increased with HC11 cell differentiation, we examined whether Stat3 activity might, in fact, be required for differentiation. Three Stat3 inhibitors (CPA7, SH4-54, S3I-201) were employed at low levels, as to induce an inhibition of 15-20% (see Materials and Methods). As shown in Figure 2A, Stat3 inhibition using CPA7 eliminated differentiation, as revealed by β -casein levels (lanes 1-19).

Similar results were obtained with the SH4-54 (lane 21) and S3I-201 (lane 22) inhibitors, in agreement with previous data (18). Together with the demonstration that E-cadherin is required for HC11 differentiation (13), these findings point to the possibility that Stat3 is transducing the differentiation signal emanating from E-cadherin in this system.

As shown in Figure 1, a slight decrease in Stat3-ptyr705 levels was consistently observed during days 6-10 post differentiation induction. To examine the requirement for Stat3 at later times along the differentiation process, CPA7 was added on day 5 following differentiation induction. As shown in Figure 2B, CPA7 addition on day 5 could reduce

but not eliminate casein levels, compared to its addition at the beginning of the differentiation process (Figure 2A, lanes 1-19), while addition on day 7 or beyond had no effect. These data strongly suggest that Stat3 activity is needed specifically during the early stages of differentiation, while it may be dispensable once the differentiation process is underway.

Constitutively active Stat3C inhibits HC11 cell differentiation in the absence of full neoplastic transformation. Since Stat3 is required for, and is up-regulated during breast epithelial cell differentiation, it might be expected that its constitutively active mutant, Stat3C, would reinforce or potentiate the Stat3-mediated differentiation signal. To examine the effect of Stat3C, the latter was stably expressed with a retroviral vector [clones MSCV-Stat3C-a and -b (20), Figure 3A]. Interestingly, Stat3C, when expressed to levels similar to the endogenous Stat3 in clones MSCV-Stat3C-a and -b, it was able to eliminate differentiation of HC11 cells (Figure 3A).

To further verify the effect of Stat3C on HC11 differentiation, Stat3C was expressed using a lentiviral vector and clones expressing very low Stat3 levels (~15% Stat3 transcriptional activity compared to the control HC11-Stat3C, clones lenti-Stat3C-c and -d) were chosen for further analysis. As shown in Figure 3B, differentiation was reduced, although not eliminated. Taken together, the above data reveal an inverse, quantitative relationship between differentiation and Stat3 activity levels (Figure 3C). Unlike mutationally activated, Rac^{V12}, which at low levels could increase differentiation, no increase in differentiation was ever observed in the Stat3C-expressing clones examined, as it would have been expected in the case where Stat3C potentiated the E-cadherin-mediated differentiation signal.

To examine whether the inability of Stat3C-expressing HC11 cells to differentiate could arise from their transformed state, we examined their phenotype regarding their capacity to grow in an anchorage-independent manner. Stat3C-expressing cells were suspended in soft agarose with HC11-Stat3C cells as a positive control, and their colony-forming ability was examined. As shown in Figure 4, HC11-Stat3C cells formed colonies in soft agarose. However, the colony-forming ability of MSCV-Stat3C or lenti-Stat3C clones was not higher compared to the parental HC11, or cells expressing GFP alone (13). Therefore, despite the fact that the Stat3C levels expressed in the MSCV-Stat3C-a and b clones tested were sufficient to reduce differentiation to undetectable levels, they were not able to promote anchorage-independent proliferation (Figure 4). Taken together, the above results demonstrate that Stat3C is unable to substitute for the E-cadherin/Rac-mediated differentiation signal. On the contrary, Stat3C blocks differentiation. Moreover, Stat3C at very low levels that is unable to fully transform HC11 cells, is still able to block their

differentiation, indicating that this ability is not derived from, and can be clearly uncoupled, from its transforming effect.

Discussion

Mammary epithelial cell differentiation depends on the coordination of a complex array of extra- and intracellular signaling molecules. This work examined the role of E-cadherin/Stat3 signalling on HC11 mammary epithelial cell differentiation as opposed to neoplastic conversion, in an attempt to uncover the consequences of dysregulation of this pathway upon cell fate.

Stat3 activity is required for HC11 mammary epithelial cell differentiation. Since confluence, which dramatically increases levels of Stat3-tyr705 (10), is a known prerequisite for epithelial cell differentiation (2), we explored the Stat3 activity requirement for the differentiation of HC11 cells. Our results demonstrated an increase in Stat3 activity with HIP treatment. Indeed, Stat3 has been identified as an effector of the prolactin receptor (26, 27), therefore prolactin may be the element responsible for the observed Stat3-tyr705 elevation in HIP treated cells.

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Figure 3. *Constitutively active, Stat3C reduces HC11 cell differentiation.* (A) *MSCV-Stat3C expression eliminates HC11 cell differentiation:* HC11 cells expressing Stat3C with the MSCV retroviral vector (clones MSCV-Stat3C-a and MSCV-Stat3C-b), as well as the parental HC11 cells, were grown in the absence of EGF for 24 hours, then they were induced to differentiate with HIP addition for the indicated number of days (lanes 3-6, 9-12 and 15-18). Cells treated with medium lacking EGF (NE) served as controls (lanes 1-2, 7-8, and 13-14). Detergent cell extracts were probed for β -casein, total-Stat3, or β -actin as a loading control, as indicated. Representative of 3 experiments. Numbers at the right refer to molecular weight markers. Note the lack of β -casein expression in MSCV-Stat3C-a or MSCV-Stat3C-b cells compared to the parental HC11 (lanes 9-12, 15-18 versus 3-6). (B) *Lentiviral Stat3C-GFP expression reduces HC11 cell differentiation:* HC11 cells expressing Stat3C with a lentiviral vector (clones Stat3C-c and Stat3C-d), as well as parental HC11 cells, were grown in the absence of EGF for 24 hours, and were then induced to differentiate with HIP addition for the indicated number of days. Detergent cell extracts were probed for β -casein, total Stat3, or β -actin as a loading control, as indicated. Numbers at the right refer to molecular weight markers. Representative of 3 experiments. Note the reduction of β -casein expression in HC11-Stat3C cells compared to the parental HC11 (lanes 5-8, 9-12 versus 1-4). (C) *Stat3 transcriptional activity following Stat3C expression:* i) *Upper panel:* HC11 cells, before or following Stat3C expression as above, were transfected with the Stat3-dependent pLucTKS3 and the Stat3-independent pRLSRE reporters at a confluence of 30%. 48 hours later, firefly (■) or Renilla (□) luciferase activities were determined in detergent cell extracts (23). Values shown represent luciferase units expressed as a percentage of the highest value obtained, means $\pm$ SEM of at least 3 experiments, each performed in triplicate. ii) *Lower panel:* Same cells as above, probed for β -casein to assess differentiation.

Figure 4. Anchorage-dependence of non-differentiating Stat3C-expressing cells. (A) MSCV-Stat3C-a (b), lenti-Stat3C-c (c) cells, HC11-Stat3C (d(10)), or parental HC11 (a) cells plated on soft agarose and photographed 20 days later under phase-contrast illumination. Scale bars: 100 μ m. Representative of 3 experiments. (B) Agar growth quantitation. Colonies were counted and growth was expressed as a % of total cell count. Averages of 3 experiments $\pm$ SEM.

The surge in Stat3-tyr705 levels in differentiation-induced HC11 cells prompted us to assess whether Stat3 activity might actually be required for HC11 differentiation. In fact, Stat3 inhibition by just ~15% and for just 24 h prior to the induction of differentiation eliminated their differentiation, pointing to a Stat3 requirement in this process. However, Stat3 inhibition on day 5 had a less profound effect. This result demonstrates that Stat3 activity is vital for a narrow window of time, during early stages of differentiation, while after this point Stat3 activity appears to be dispensable.

Stat3 has been demonstrated to provide a potent survival signal, which is especially important at confluence and later time points [(10, 28), reviewed in (25)]. However, the fact that HC11 differentiation is sensitive to the timing of CPA7 treatment, points to alternative mechanisms. In fact, Stat5, another member of the family, is a well-documented regulator of lactogenic differentiation and positively regulates the β -casein promoter (27). As Stat3 and Stat5 have been shown to form heterodimers (29) and are active in this system, this could suggest a way in which Stat3, through its interaction with Stat5, might transcriptionally up-regulate the

β -casein promoter and therefore contribute to β -casein expression and differentiation.

Expression of constitutively active, Stat3C reduces mammary epithelial cell differentiation. Although Stat3 activity is required for HC11 cell differentiation, our results indicate that overexpression of Stat3C was neither able to drive the expression of the differentiation marker, β -casein, in uninduced cells, nor to potentiate the differentiation signal in HIP-induced cells. On the contrary, all HC11-Stat3C clones either display a markedly reduced expression of β -casein or they fail to differentiate altogether.

Since Stat3C can act as an oncogene, and a number of oncogenes are known to block differentiation, we examined whether this inhibition of differentiation could be attributable to the activation of oncogenic targets that may be dominant over the pro-differentiation signals. However, our results revealed an absence of measurable growth in agarose. This could be due to the fact that the Stat3C levels used in the clones were not sufficient for transformation, despite the fact that they suppressed differentiation. In any event, the inability of the cells

to grow in the absence of anchorage suggests that the block of differentiation by Stat3C is not immediately attributable to its transforming potential. Still, the fact that no increase in β -casein expression was observed even at very low levels points to differences in the effectors of Stat3C *versus* Rac^{V12}, which promote differentiation. It is conceivable that even lower Stat3C levels might have increased differentiation, as in the case of Rac^{V12}, however, no increase in the differentiation was ever observed, even when Stat3C levels were barely detectable.

We recently demonstrated that Rac^{V12} expression in HC11 cells is able to down-regulate E-cadherin, even at levels that are insufficient to fully suppress differentiation (13). Since Stat3 is a downstream effector of Rac in HC11 cells (10), it might be expected that Stat3C, at levels sufficient to block differentiation, might reduce E-cadherin levels. However, in sharp contrast to cells expressing even low amounts of Rac^{V12}, Stat3C was found to be unable to reduce E-cadherin levels even when expressed to levels that are sufficiently high as to eliminate differentiation (not shown). Therefore, it appears that Rac^{V12} is employing a different pathway(s) from Stat3C to block differentiation, despite the fact that Stat3 is one of the downstream effectors of Rac.

The strength of the E-cadherin/Stat3 signalling pathway is a key determinant of the outcome of the differentiation process in breast epithelial cells, with Stat3 activity being critically required for differentiation. However, overexpression of the activated form, Stat3C, rather than promoting, it blocks differentiation, even when expressed at low levels, and in the absence of full neoplastic conversion. Whether the same model holds true in other differentiation systems known to require cell to cell adhesion, such as adipocytes and myoblasts (30), remains to be seen.

Conflicts of Interest

The Authors declare that there are no conflicts of interest.

Authors' Contributions

MN, MG, ZT, RA, JC, and VH performed most experiments under the supervision of BE, PG, and LR. BE provided ideas and supervision. PG provided Stat3 inhibitors and funding. LR conceived the project, provided funding, and supervised throughout.

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